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414. EXPERIMENTS ON MILKING TECHNIQUE

7. THE EFFECT OF SUBJECTING DAIRY COWS, FOR A COMPLETE LACTATION, TO A RIGID CONTROL OF THE DURATION OF MILKING

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(With 10 Figures)

OBJECT

In earlier communications (1, 2, 3) we have reported attempts to alter the rate of milking of dairy cows by changes in milking management. These attempts failed to produce a major effect, and the changes induced were very small compared with the large differences in rate of milking between individual cows. These experiments were short-term and the cows had previously been conditioned to other milking routines and, in consequence, may have acquired particular milking habits before going on experiment. In the experiment reported below, all the animals were in their first lactation, and the treatments were enforced from the first day that milking machines were used until the cows became dry at the end of the lactation.

The primary object of the experiment was to find the effect on milking rate of subjecting the cows to a fixed milking time of either 4 or 8 min. at every milking.

METHOD

The experiment was carried out on four farms in Berkshire between July 1947 and March 1949. All herds were attested, and in each the standard of management was well above the average. Table 1 gives the details of the farms, the breed and numbers of the animals used and the type and characteristics of the milking machines.

Table 1. *Details of cows and milking machines used*

Farm	Breed of cow	No. of cows on experiment	Type of milking machine	Type of teat cup liner	Vacuum (in. Hg)	Pulsation rate
A	Guernsey and Shorthorn	12	Bucket	Moulded	15	42
B	Shorthorn	6	Bucket	Moulded	13	45
C	Shorthorn and Friesian	6	Bucket	Moulded	14	60
D	Ayrshire	16	Bucket	Moulded	15	54

In all, twenty-two Shorthorns, sixteen Ayrshires and two Friesians were used, but because of an accident to one cow a pair of Shorthorns had to be eliminated and a total of thirty-eight cows completed the experiment. The milking routines on the four farms were as follows:

Farm A: The cows were washed with hot water (100–120° F.) about 1–2 min. before milking. Concentrated foods were fed at least 20 min. before the start of milking.

Farms B and C: All the cows were washed at least 15 min. before milking, and had been conditioned to accept the feeding of concentrated foods immediately before milking as a 'let down' stimulus.

Farm D: The cows were fed their concentrate ration and washed at least 15 min. before milking.

As the animals were first-calf heifers, and because of the nature of the experiment, a pre-experimental control period was not possible, and therefore the animals could not be paired for milk yield or milking rate. The heifers were treated in pairs as they calved, the treatment being decided by lot. Each animal was subjected to the treatment from the first milking until drying-off. To ensure a regular and accurate enforcement of the time schedules the milkers were provided with either a stopwatch or a clockwork timing device. In spite of the fact that some heifers which were milked for 4 min. were not milked completely for several months and others which were milked for 8 min. had the teat cups left on for considerably longer than was necessary, the treatments were rigidly enforced. In addition to measuring the effect of the treatments on milking rate it was possible to determine the effect on yield and quality of milk and on the incidence of mastitis.

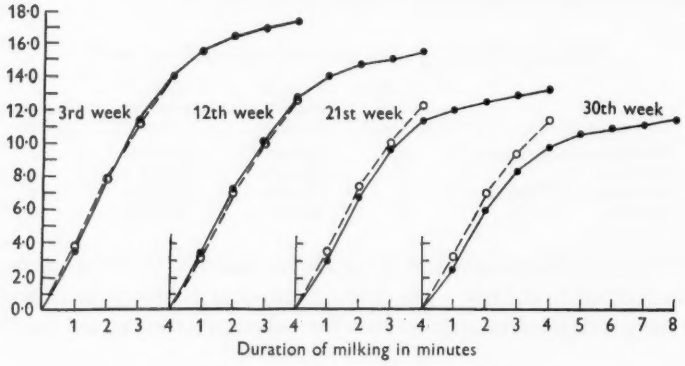
The milk yield of each cow was recorded at every milking, and samples were taken for milk quality on one day every 3 weeks, and additional samples whenever this was thought to be desirable. Milking-rate determinations and tests for mastitis were also carried out at three-weekly intervals.

RESULTS

The experimental results discussed below are illustrated graphically as mean curves for the first 30 weeks of lactation; after this date a number of cows became dry. No attempt has been made to measure the difference in response of the cows on the individual farms because of the small numbers employed.

(1) *Milking rate*

The rate at which the cows were milked was recorded at morning milkings by suspending the milking machine on a spring balance and recording the balance readings at 30 sec. intervals (4). The mean quantity-time graphs for both groups of heifers recorded in the third, twelfth, twenty-first and thirtieth weeks of lactation are given in Fig. 1. These



○ – – ○ = '4 min.' group. ●—● = '8 min.' group.

Fig. 1. Mean quantity-time graphs: for the two groups of cows.

graphs clearly indicate that there was no major difference in milking rate between the two groups. In the third and twelfth weeks the graphs appear to be almost identical for the

first 4 min., though on the later two occasions the '4 min.' group is slightly faster, especially in the fourth minute of milking. This may have been due, in part, to manipulation of teat cups on the '4 min.' cows by the milkers during the last minute of milking.

A more detailed examination of the milking rates is given in Figs. 2–5, which show, for the two groups, the mean graphs for peak flow (i.e. the highest rate of milking attained), the yield of milk in the first minute after the teat cups were put on the cows, the rate of milking in the second and third minutes, and the interval between the start of milking and the end of the minute when the fastest rate was attained.

A major factor in determining the normal duration of milking is the peak flow; clearly this was not greatly affected by the treatments (see Fig. 2). Figs. 3–5 show that there were minor differences in milking rate between the two groups, at least for the first 20 weeks of lactation. It appears that the cows on the '4 min.' treatment gave more of their milk in the first minute, less in the second and third minute and generally reached their peak rate of milking earlier than the '8 min.' cows. These differences are not statistically significant, and even if they are due to the experimental treatments they are small compared with the changes necessary to milk a slow-milking cow completely in 4 min.

(2) *Yield and chemical quality*

Fig. 1 shows that on average the main difference between the two treatments was that in the '4 min.' group the machines were removed while the milk was in full flow, whereas in the '8 min.' group the machines were left on long after the milk had ceased to flow. Palpation of the udder of the cows after the removal of the machines indicated that about half of the '4 min.' group were incompletely milked for a considerable period after calving, though the proportion declined with advance in lactation (Table 2).

Table 2. *The number of cows in the '4 min.' group which were indicated by udder palpation to be incompletely milked when the machines were removed from the cows*

Weeks	3	6	9	12	15	18	21	24	27	30
Total cows	19	19	19	19	19	19	19	19	19	19
No. incompletely milked	10	9	10	10	9	9	8	6	5	4

This incomplete milking is more objectively illustrated in Fig. 6, where graphs of the mean ratio of morning to evening milk yields for the two groups are presented. Normally, the ratio of morning to evening milk yields is similar to the ratio of the intervals between the milkings, the similarity being closer in later lactation when udder capacity does not limit milk production. From the graph it can be seen that the ratio of morning to evening milk yield in the '4 min.' group is much nearer unity, a difference which is significant ($P < 0{\cdot}05$) for the first 24 weeks after calving. The duration of milking and not the intervals between milkings was the factor limiting milk yield at each milking.

According to current beliefs it might be expected that this incomplete milking for a considerable period of the lactation would reduce milk yield, fat percentage and persistency. The effect in this experiment is shown in Figs. 7–10, where the mean weekly milk yield, mean daily fat yield, fat percentage and solids-not-fat percentage are shown. From these graphs it would appear that the '4 min.' treatment had an effect on milk and fat yield and fat percentage in early lactation corresponding to the period when most milk was left in the udders. There was no effect on the solids-not-fat percentage. A simple statistical analysis was carried out on the effect of the treatment on the total yield of milk, yield of

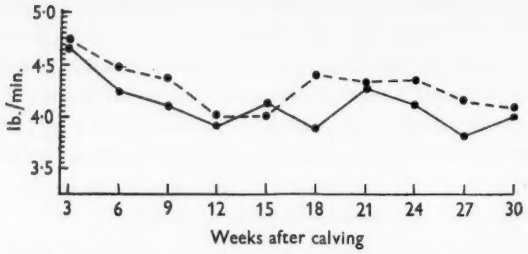


Fig. 2. The mean 'peak flow' rates of the two groups of cows.

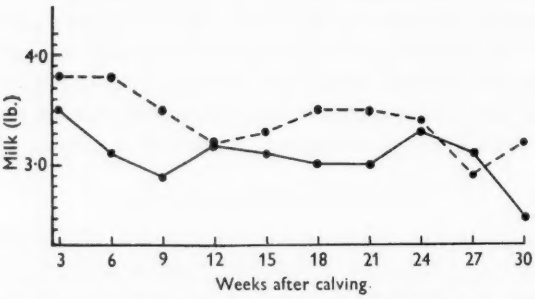


Fig. 3. The mean yields of milk obtained in the first minute of milking of the two groups of cows.

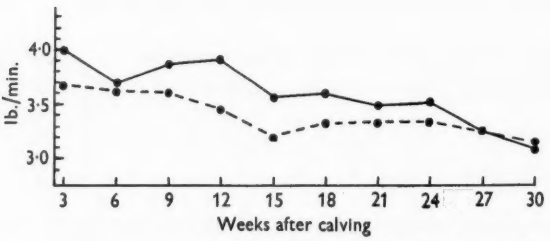


Fig. 4. The mean rates of milking in the second and third minutes of milking of the two groups of cows.

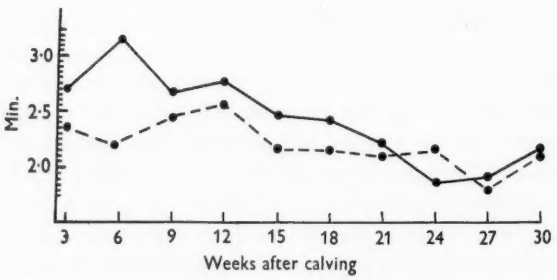


Fig. 5. The mean duration of the period between the start of milking and the end of 'peak flow' for the two groups of cows.

In figs. 2–10, ●——● = '8 min.' group and ●- - -● = '4 min.' group.

fat and the fat percentage up to the twenty-first week after calving. Though the effect on milk yield over the whole of this period was non-significant, the reduction in fat yield and fat percentage in the '4 min.' group was significant ($P < 0{\cdot}05$). The difference in milk yield between the two groups was significant ($P < 0{\cdot}05$) up to the sixth week after calving.

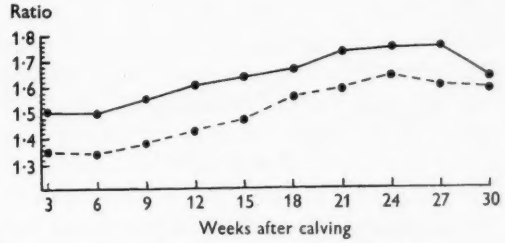


Fig. 6. The mean ratios of the morning milk yield divided by the evening milk yield for the two groups of cows.

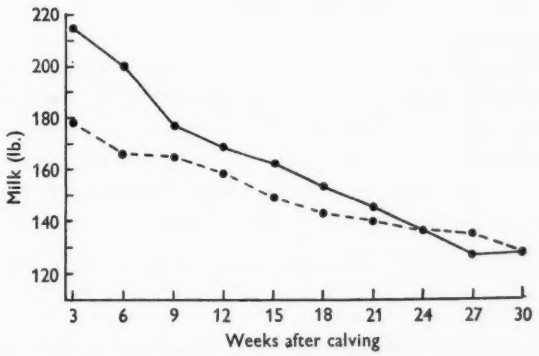


Fig. 7. Mean weekly milk yield (lb.) of the two groups of cows

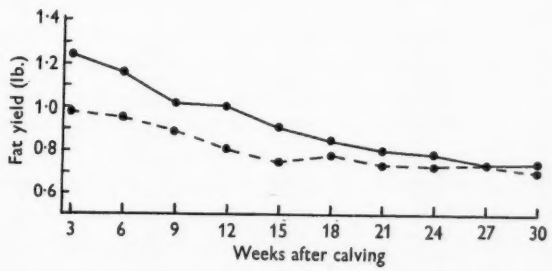


Fig. 8. The mean daily yield of fat of the two groups of cows.

In Table 3 the uncorrected 305-day yields of milk and butterfat for the two experimental groups are given. Though the mean yields of milk and fat of the cows on the '8 min.' treatment are 187·5 and 19·2 lb. greater than those on the '4 min.' treatment, these differences are not significant. A further analysis made on the twelve pairs of cows free from clinical mastitis, for the whole lactation, again showed no significant treatment effect.

The lactation yields were not corrected because the mean ages of the '4 min.' and '8 min.' groups on calving were 1095 and 1098 days respectively, and the mean intervals between first and second calvings were 402 and 396 days respectively.

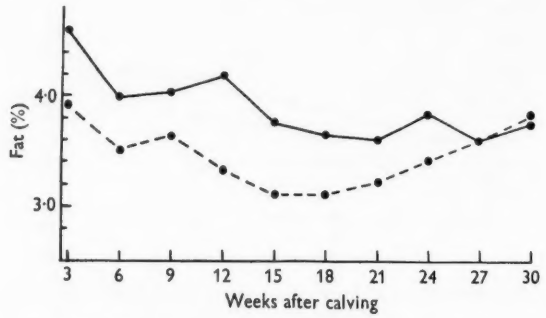


Fig. 9. Mean fat percentage of the two groups of cows.

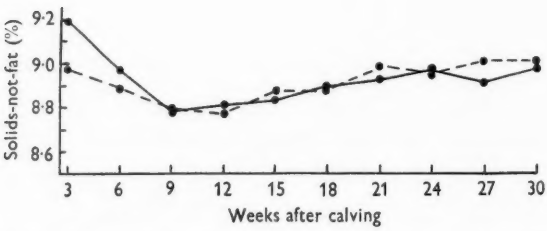


Fig. 10. The mean solids-not-fat percentage of the two groups of cows.

Table 3. *The uncorrected* 305*-day yields of milk and fat from the two groups of cows*

	Milk (lb.)		Fat (lb.)	
Treatment ...	'4 min.'	'8 min.'	'4 min.'	'8 min.'
Pair no.				
1	2954*	6482	124·1*	344·8
2	4478†	6055	180·5†	254·3
3	5419	4052*	217·3	149·9*
4	4818†	4851*	173·9†	218·8*
5	6208	4908	255·1	186·0
6	4533	5733	186·8	229·9
7	7016†	5816	211·2†	251·3
8	7963†	4559*	311·4†	150·5*
9	7998	5262*	320·7	189·5*
10	5858	6163	241·6	258·9
11	6520	8976	249·1	333·1
12	4586†	6473	143·0†	240·8
13	6842	8565	252·8	362·3
14	5415†	5540	201·4†	230·5
15	5238†	7266	168·5†	270·3
16	6341†	6591	223·7†	240·4
17	5946	6448	221·2	258·6
18	6424†	6426	271·7†	231·9
19	6090	4044	208·3	125·4
Means	5823·5	6011·0	219·1	238·3

* Cows clinically affected with mastitis.

† Cows incompletely milked for at least 6 months of the lactation.

Because the heifers could not be paired for yield or speed of milking before the start of the experiment there is a very large within-pair variation for both these factors. This accounts to some extent for the lack of significance in these differences discussed above.

(3) *Mastitis*

Though the heifers used in this experiment were in each case part of a larger group of cows, they were always segregated, to some degree, from the rest of the herd. In herds A, B and C the heifers were in the same cowshed as the rest of the herd, though on each farm the experimental heifers stood together. The heifers at farm D were completely separate from all other animals. In herds A and D the heifers were milked as a group with machines that were not used for other cows, whereas in herds B and C, for a short period at the end of the lactation, they were milked with machines that had been used on other cows. On all farms steam was used to sterilize the machines at least once a day, and the maintenance and cleaning of the machines was good. Udder washing was with water containing sodium hypochlorite in herds B, C and D, and in herd D a separate sterile cloth was used for each cow. None of the farms practised teat-cup dipping in hypochlorite. Clinical mastitis was not common in any of the four herds. Herd D was known to be free from *Streptococcus agalactiae* mastitis, and this organism was not recovered in any sample from herds B and C.

At each of the visits made to the farms at 3-weekly intervals the following tests and udder examinations were made. The foremilk of all cows was examined for clots and discoloration, and the teat orifices were examined for erosions. After careful disinfection of the teats with hypochlorite one foremilk sample was taken from each quarter. These samples were usually tested shortly after collection, though some were refrigerated overnight and tested the following morning. About 0·05 ml. of the milk was plated on the surface of freshly poured plates of aesculin blood agar and the plates were incubated at 37° C. All colonies on the plates were counted; streptococci were identified by biochemical and serological methods. Staphylococci were distinguished from micrococci by colony appearance, haemolysis and by the slide coagulase test with human plasma.

The modified Whiteside test as outlined by Murphy & Hanson (5) was carried out on all foremilk samples.

Quarters were diagnosed as infected by the presence of pathogenic bacteria on two or more consecutive tests, where the milk samples also showed a reaction to the Whiteside test. It is realized that a number of temporary infections may have been missed, but the chance of this occurring was equal for the two groups of animals. It is interesting to note that in all cases where pathogenic bacteria were recorded, and the Whiteside result was also positive, confirmation was obtained at the next test. A quarter was regarded as clinical if it was inflamed or indurated or the milk was discoloured or contained clots. Three of the five cows showing clinical symptoms were treated with penicillin successfully, while two, not treated, lost their clinical symptoms but remained infected.

Results of the experimental treatments on mastitis incidence are shown in Table 4.

These bacteriological and clinical data indicate that there was more mastitis in the cows submitted to the '8 min.' treatment. A χ^2 test carried out on the numbers of quarters infected and clinical reveals that this difference is significant ($P < 0{\cdot}05$). Presumably the reason for this higher incidence was that the machines were left on the cows longer than was necessary to milk them and that this in some way damaged the udder, making the cows more susceptible to attack. From the results the precise way in which the udders

were injured is not obvious. The examination of teat orifices at 3-weekly intervals revealed that five cows on each treatment showed persistent erosion. Clearly treatment had not played a serious part in eroding the teat orifices. It appeared that certain cows had teats that were liable to erosion whatever the treatment. It is interesting to record that all the four quarters of these cows were affected to some degree and that most of them were slow milkers. Of the twenty-four heifers with a maximum milking rate of over 4·0 lb./min. in early lactation, only one suffered from erosion of the teats, whereas nine of the fourteen cows with a milking rate of less than this had erosions. Though five out of the ten cows with eroded teats on both treatments became infected, whilst only six of the remaining twenty-eight became infected, these differences in infection rate are not significant.

The results of the Whiteside tests are given in Table 5, where the total Whiteside score for the twelve pairs of heifers remaining uninfected throughout are given.

Table 4. *The numbers of cows and quarters infected in the two experimental groups and the numbers becoming clinical*

	'4 min.'	'8 min.'
No. of cows	19	19
No. of cows infected	4*	7
No. of cows clinical	0*	4
No. of quarters infected	4*	16
No. of quarters clinical	0*	8
No. of cows with eroded teats	5	5

* One cow on the '4 min.' treatment received an injury to a teat which resulted in a clinical attack. As the injury was clearly not due to the treatment the results of this infection have been omitted.

Table 5. *Results of the Whiteside tests of the twelve non-infected pairs for the first ten 3-weekly tests*

	Total Whiteside score for the 12 heifers*									
Three-weekly periods	1	2	3	4	5	6	7	8	9	10
'4 min.' group	1	1	3	3	5	6	5	2	4	23
'8 min.' group	0	2	2	2	9	6	3	5	23	13

* The milks were graded on the results of the tests and the grades were given the following score: ± =1, 1+ =2, 2+ =4, 3+ =6 and 4+ =8. Each number in Table 5 represents the total score of the twelve heifers.

These results do not suggest that there was much udder irritation caused by either treatment; the only significant change occurred equally in both groups when the readings increased with the decline in yield in late lactation.

The theory that incomplete milking predisposes cows to clinical mastitis is not substantiated. In spite of the fact that half of the '4 min.' group had milk left in their udders at the end of each milking for the major part of their lactations, the infection rate of this group over complete lactations was as low as 5% of the quarters.

DISCUSSION

In recent years the view has commonly been held that milk yields can be increased if cows are 'trained' to milk more quickly by machine. There appears to be little published evidence that cows which can be milked quickly are also milked more completely, or that they give more milk than slow-milking cows under similar conditions. This supposition, however, appears to be logical if the current neuro-hormonal theory of milk 'let-down' in the udder is accepted. However, this is of little immediate commercial significance if, in

fact, it is not practicable to train a slow-milking cow to milk at a faster rate. The results of the experiment described above, which covers complete lactations, illustrates once more a failure to bring about major changes in milking rate by restriction in duration of milking. This confirms earlier findings (1,2) where restrictions were imposed over shorter periods. Minor variations in milking rate can be brought about by changes in methods of using the same machine at the same vacuum level(3,7), but these may be due to little more than variations in udder pressure or changes in the amount of milk left in the udder after milking. These individual changes in milking rate are very small compared with the great differences between those of individual cows milked under similar conditions.

Some interesting incidental data on the effect of leaving milk in the udder were also obtained in the experiment described. Fully half of the heifers on the 4 min. treatment did not milk out completely in the time allowed, and consequently, for much of the lactation, there was milk left in the udder after each milking. The fall in milk yield was surprisingly small, and the effect on fat secretion was significant only in early lactation and there was no reduction in persistency. Apparently, the reduction in yield which did occur in early lactation was due to the milk left behind reducing internal udder capacity.

After this early period the average persistency of the cows in the 4 min. group was fully as high as those in the 8 min. group, the mean length of lactation being 315 and 303 days respectively.

The reduction in fat percentage in the 4 min. group was contrary to the findings of Johansson (8), though his results were obtained in short-term experiments. However, we can suggest no reason for the greater inhibition of fat secretion compared with the secretion of the non-fatty fraction of milk.

From the results concerning mastitis it appears that under the conditions of management found on these farms there was a greater incidence of clinical and subclinical mastitis when the machines were left on the cows for 8 min. at each milking. There was no difference in the incidence of teat erosions between the two groups neither was there any difference in the Whiteside test results of the non-infected cows in each group. This indicates that although the 8 min. group was susceptible the infections did not follow obvious teat injury or marked rises in the leucocyte counts of the milk. Contrary to the findings of Schalm & Mead (9), incomplete milking had no serious measurable effect on the incidence of clinical or subclinical mastitis; in fact, the 4 min. group had a low incidence rate and no clinical mastitis at all. It is, however, important to emphasize that this experiment was carried out using cows in their first lactation that are less susceptible than older cows. Furthermore, the management of the cows, the hygiene and mastitis control, were very efficient in all herds, and hence the degree of exposure was low. It is probable that the mastitis incidence in the 8 min. group of animals would have been higher if the experiment had been carried out in a herd with poor hygiene and a high incidence of *Str. agalactiae* mastitis.

SUMMARY

1. Thirty-eight first-calf heifers in two equal groups were subjected for complete lactations to a rigid control of the duration of milking to either 4 or 8 min.

2. The treatment had no major effect on the milking rates of either group.

3. Whilst 4 min. was insufficient time to milk out completely the udders of about half the animals on this treatment, the machines on the 8 min. treatment were generally left on for a period after milk flow had stopped.

4. There was no significant difference between the 305-day lactation yields of the two groups or in the persistency. The 4 min. treatment did, however, decrease the yield of milk and fat in early lactation.

5. The 8 min. cows had more clinical and subclinical mastitis, though they did not have a higher proportion of eroded teats nor higher Whiteside test readings.

6. Teat erosion appeared to be correlated with slow milking.

We wish to express our sincere thanks to Prof. H. G. Sanders, of Reading University, Dr W. S. Gordon, Director of the Agricultural Research Council's Field Station at Compton, and Dr A. H. Lewis, Director of the Imperial Chemical Industries Field Station at Jealotts Hill, for their kind co-operation in placing heifers at our disposal for complete lactations in order to carry out the work described above.

We are also indebted to Dr S. J. Rowland for carrying out chemical analyses of the milk samples, to Mr C. P. Cox for help with the statistical analyses and to Miss E. S. Baxter for preparation of data for publication.

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415. FACTORS AFFECTING THE RATE OF MACHINE MILKING

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(With 5 Figures)

INTRODUCTION

It has recently been shown that there are very wide differences between cows in the inherent rate of milking by machine, while the variations in the same cow within a lactation and between lactations are usually quite small (1, 2). There appears to be little published evidence on the causes of the big differences in milking rate between cows.

Smith & Petersen (3) have shown that the rate of milking was increased by increasing the vacuum at which the milking machine was operated. Whittleston & Verrall (4) failed to confirm this, although their experiment was stated to be not of a high order of precision.

The object of the short-term experiment reported below was to investigate the factors controlling milking rate, in particular, the parts played by the vacuum and the size of teat orifice.

METHODS

The cows used in this experiment were milked from calving, and throughout the experiment, by one of the individual quarter milking machines described by Hall (5). This machine delivered the milk from the teats through separate tubes to four Perspex cylinders which were graduated in 1 lb. and $\frac{1}{10}$ lb. divisions and maintained under vacuum during milking. The vacuum could be altered by changing the weights on a weight-loaded relief valve. For this experiment three levels of vacuum* were used, 10·6, 15·9 and 20·4 in. Hg which, for simplicity, will be referred to in the text as 11, 16 and 20 in. Hg. The pressures were checked with a mercury manometer at intervals throughout the experiment.

At each milking the cows followed a fixed routine. They entered the bail, received their concentrated foods and had their udders washed with warm water. Within a minute of the completion of washing the teat cups were put on. The animals soon became accustomed to this routine; they readily entered the bail, and no discomfort was shown under any of the milking machine treatments used.

By inserting a simple cannula into the teat and connecting it to the lid of one cylinder by the normal milk tube, it was possible to measure the rate of milking of each quarter at each level of vacuum through an orifice of a size determined by the bore of the cannula. During the experimental milkings the cannula was used on one hind quarter, whilst the other three quarters were being machine milked in the normal way with teat cups. It was hoped thus to ensure similar conditions for 'let-down' in the cannulated and machine-milked quarters. The same cannula was used throughout the experiment.

To test whether the capacity of each milk tube was sufficient to carry the maximum flow from the fastest quarter, the whole teat-cup assembly was previously inverted in a bucket

* The term 'vacuum' is used to denote the reduction in pressure below atmospheric pressure and is quoted in inches of mercury.

of water and the rate of flow measured. For each cylinder this was approximately 16·2 lb./min. at the 20 in. Hg vacuum, which was more than four times faster than the fastest milking quarter at this vacuum. It was also established that with this machine the vacuum at the base of the teat cup did not fall by more than 1 in. Hg when the fastest cow was being milked at a vacuum of 20 in. Hg.

By connecting the cannula to the base of a glass cylinder, $1\frac{1}{2}$ in. in diameter, containing a column of warm milk (90–100° F.), the rate of flow through the cannula, at the various levels, was tested. These rates when the column of milk in the reservoir was at 3 and at 5 ft. are given in Table 1.

Table 1. *Flow of milk through cannula from reservoir (lb. per min.)*

Height of column (ft.)	Atmospheric pressure	Vacuum (in. Hg) 10·6	15·9	20·4
3	0·95	2·15	2·60	2·90
5	1·22	2·30	2·73	2·98

The data given in Table 1 and data in subsequent tables, referring to rate of flow from the teat, in fact apply to rate of collection in the cylinder, which is not precisely the same as the rate of flow from the teat. Since, however, the difference was approximately constant throughout the experiment, it is considered unlikely that the validity of comparisons between treatments was affected.

EXPERIMENTAL DESIGN

The four cows used in this experiment were all in early lactation, and their yields were in the range 35–50 lb. per day. The experimental milkings were carried out at alternate morning milkings for 15 days, giving eight experimental readings for each cow. Speed-of-milking records were collected from the two hind quarters of each cow on each day of the experiment. One quarter was milked by teat cup and the other through the cannula. At alternate morning milkings and at all evening milkings the cows were milked with the

Table 2. *Design of experiment*

Method of milking	Treatments: Vacuum (in. Hg) A	B	C	D
Cannulation	0	10·6	15·9	20·4
Machine	15·9	10·6	15·9	20·4

Days are numbered serially, the numbers referring to the morning milking of the days on which treatment was given. t = cannulation; m = teat cup milking.

	Day ...	1	2	3	4	5	6	7	8
Cow	Hind Quarter								
1	Left	Am	Ct	At	Cm	Bt	Bm	Dm	Dt
	Right	t	m	m	t	m	t	t	m
2	Left	Cm	At	Bm	Am	Dt	Dm	Ct	Bt
	Right	t	m	t	t	m	t	m	m
3	Left	Dt	Bm	Dm	Bt	Am	Ct	At	Cm
	Right	m	t	t	m	t	m	m	t
4	Left	Bt	Dm	Ct	Dt	Cm	At	Bm	Am
	Right	m	t	m	m	t	m	t	t
Latin square:		I	II	II	I	I	II	II	I

standard machine operated at a vacuum of 16 in. Hg, so that there were three control milkings between each of the experimental recordings. At the experimental milking the vacuum applied was the same for both hind teats of a cow, except where the cannulation milking was at atmospheric pressure, when the teat-cup milking was carried out at a vacuum of 16 in. Hg. The pulsation rate was kept at 40 per min. throughout; there was no pulsation on a quarter which was milked by cannula.

Table 2 illustrates the experimental design which was in the form of two 4×4 Latin squares with split rows, the squares being complementary half-replicates. Columns were determined by days, rows by cows, and the split-plot comparisons were between milking by teat cup and by cannula. Columns of the Latin squares were interlaced, and in each square one degree of freedom for the interaction vacuum pressures *v.* methods of milking was confounded with the difference over all cows between left and right quarters.

COLLECTION OF DATA

On each of the eight mornings when experimental data were collected the milking rate of each hind quarter of each of the four cows was recorded. The quantity of milk was noted at intervals of 30 sec. throughout the milking. Machine stripping of the hind quarter milked by teat cup in each cow was started when the yield of milk in 30 sec. fell to 0·1 lb. or less, and the stripping was continued until the quarter was cleanly milked. The cannulated quarters was not stripped in any way, and as soon as milk ceased to flow the cannula was removed. Except when the cannula was operated at atmospheric pressure, flow in the cannulated quarter ceased before the other hind quarter required machine stripping. The final reading from each cylinder gave the total yield from each hind quarter.

In a preliminary trial considerable trouble was experienced with foaming in the cylinder, especially with the fast-milking cows at the high levels of vacuum. To overcome this difficulty 2 ml. of amyl alcohol was added to each cylinder.

RESULTS

Several factors have been extracted from the data for separate analysis in order to consider different aspects of milking rate. These were:

(1) Peak rate of flow (lb./min.): the maximum milking rate attained over any minute during milking.

(2) Machine milking rate (lb./min.): the yield obtained before the start of stripping divided by the time taken to reach this stage.

(3) Overall rate (lb./min.): the total yield divided by the total duration of milking including stripping in teat-cup milking.

(4) Yield of strippings (lb.): the yield of machine strippings.

(5) Total milk yield (lb.).

Statistical analyses were carried out on peak rate of flow for milking both by cannula and teat cup, and on the yield of strippings and total yield for teat-cup milking.

From the observations there was evidence that for the peak rate of flow, where comparison of teat-cup milking and cannulation was required, the error variance was less for cannulated milking than for teat-cup milking, and instead of the usual Latin square analysis separate analyses were made for the two methods of milking.

One of the seven degrees of freedom for days was completely confounded with the differences between quarters within cows. Constants were fitted for the remaining six

degrees of freedom for days, the four degrees of freedom for quarters within cows and the nine degrees of freedom for the interaction of cows with level of vacuum.

Two sets of values were missing, one for teat-cup milking and one for cannulated milking. These were estimated by the mean values for the appropriate level of vacuum except in the case of peak rate of flow for teat-cup milking, where the value for the duplicate treatment (16 in. Hg.) on the same quarter was taken.

The individual values, the means and the standard errors are given in Tables 3-6; Appendix I shows the mean squares in the analyses of variance.

The values in the tables of means should not, as they stand, be used to estimate the significance of differences between quarters, or of interactions of cows with levels of vacuum, since the mutual non-orthogonality of days, quarters and cows *v.* levels of vacuum invalidates such direct comparisons. The significance of these effects may be tested by the mean squares in the analyses of variance.

Table 3. *Peak rate of flow* (*lb./min.*)

(*a*) Milked by cannula

Cow	Quarter	Vacuum (in. Hg) 0	10·6	15·9	20·4	Mean (±0·053)	L. – R.
1	Left	0·90	2·50	2·70	3·20	2·24	0·18
	Right	1·00	2·00	2·60	3·00		
2	Left	1·00	2·40	2·90	2·90	2·25	0·10
	Right	0·95	2·20	2·60	3·05		
3	Left	1·00	2·35	2·70	2·75	2·22	– 0·18
	Right	0·90	2·30	2·80	2·96*		
4	Left	1·05	2·30	2·80	2·85	2·28	– 0·05
	Right	1·00	2·40	2·85	2·95		
Mean (±0·053)		0·98	2·31	2·74	2·96	2·25	

(*b*) Milked by teat cup

Cow	Quarter	Vacuum (in. Hg) 10·6	15·9	20·4	Mean (±0·041)	L. – R.
1	Left	0·90	1·58	2·20	1·36	0·40
	Right	0·90	1·12	1·50		
2	Left	2·10	2·70	3·20	2·47	0·41
	Right	1·65	2·25	2·90		
3	Left	1·40	2·30	2·55	2·53	– 0·79
	Right	2·20	2·85	3·70		
4	Left	1·15	1·60*	1·80	1·56	– 0·04
	Right	1·25	1·55	1·95		
Mean (±0·041)		1·44	1·99†	2·49	1·98	

* Missing value estimated. † Standard error ±0·029.

(1) *Peak rate of flow*

It can be seen from Table 3 that there were very marked differences in the peak flow rates of quarters milked by teat cups even when they were milked at the same vacuum. The analysis of variance showed the differences between quarters over all levels of vacuum to be highly significant ($P < 0{\cdot}001$). For teat-cup milking the mean square for days was significant, although no clear trend or oscillation in the daily means was apparent. It is unlikely that the significance of the differences between quarters was much affected by the

confounded degree of freedom for days. On the other hand, the peak flow rates of these quarters when they were milked by cannula were all very similar, the differences between the quarters being non-significant. This is clearly illustrated in Fig. 1, where the peak-flow values of the eight quarters for both teat-cup milking and cannulation are given for the 16 in. Hg level of vacuum.

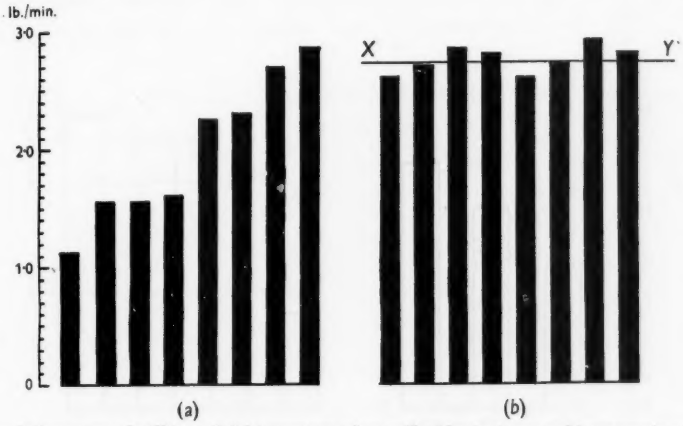

Fig. 1. The peak flow rates of milking of eight quarters when milked by teat cup and by cannula at a vacuum of 15·9 in. Hg. *XY*, the rate of flow through the cannula when fitted to a reservoir containing 5 feet of milk and operated at a vacuum of 15·9 in. Hg. (*a*) Milked by teat cup. (*b*) Milked by cannula.

Increase in vacuum gave a highly significant increase in peak rate of flow for both teat-cup milking and cannulation. The increase was linear with respect to vacuum in the case of teat-cup milking, having a slope of $b = 0{\cdot}104 \pm 0{\cdot}0059$ and quadratic for cannulation with an equation $y = 0{\cdot}974 + 0{\cdot}1586x - 0{\cdot}00295x^2$. The deviations from these regressions were not significant. These increases with increase in vacuum are illustrated in Fig. 2, where the mean peak rate of flow for each vacuum level is shown.

It is interesting to compare the mean values of peak rate of flow for cannulated quarters at each level of vacuum with the readings obtained using the same cannula fitted to the milk reservoir (see Table 1). For the 11, 16 and 20 in. levels of vacuum the means for cows scarcely differed from the rates of flow from the reservoir with milk at the 5 ft. level, although at atmospheric pressure the rate from the reservoir was significantly higher than for cannulation ($P < 0{\cdot}01$) (see Fig. 2). Using the reservoir with a 3 ft. level of milk similar results were obtained, though at the 11 and 16 in. Hg levels of vacuum the readings were significantly lower than the mean peak-flow rates from the cows at these levels of vacuum ($P < 0{\cdot}05$).

The mean peak rates of flow for the cannulated milkings were significantly higher at the three levels of vacuum than the corresponding means for normal teat-cup milking. An increase from 11 to 20 in. Hg in the vacuum gave a mean increase in peak flow for teat-cup milking of 1·05 lb./min., whereas the cannulation peak-flow rates increased by 0·65 lb./min. The difference, 0·40 lb./min., was significant ($P < 0{\cdot}01$), so that although peak rates were lower for teat-cup milking than for cannulated milking their increase per unit increase in vacuum was significantly greater.

It appears from Table 3*b* that the response to increased vacuum was greater for the fastest milking cows (2 and 3) than for the slowest (1 and 4). The difference between

the two pairs in mean response to increased vacuum (20–11 in. Hg) was 0·41 lb./min., but this includes a component of day-to-day variation. The value of the difference in response, adjusted to eliminate differences between days, was not significant at the 5% level, and this point cannot be considered established on the results of this experiment.

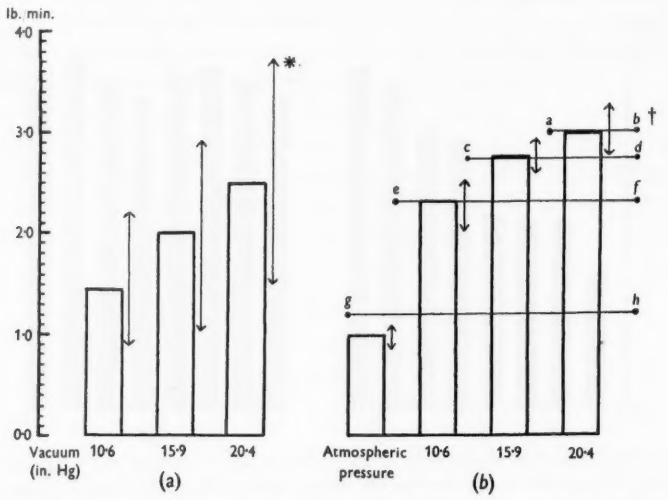

Fig. 2. The mean peak-flow rates of milking of eight quarters milked by teat cup at vacuum levels of 10·6, 15·9 and 20·4 in. Hg and by cannula at atmospheric pressure and vacuum levels of 10·6, 15·9 and 20·4 in. Hg. (*a*) Milked by teat cup. (*b*) Milked by cannula.

* The vertical arrows give the range of the values at each vacuum.

† The horizontal lines *ab, cd, ef, gh* give the flow rates when the cannula was fitted to a reservoir containing 5 ft. of milk and operated at vacuum levels of 20·4, 15·9 and 10·6 in. Hg and at atmospheric pressure.

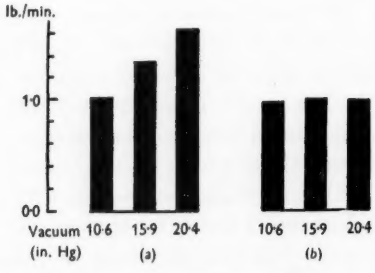

Fig. 3. The mean machine-milking rates and overall rates of eight quarters when milked by teat cup at vacuums of 10·6, 15·9 and 20·6 in. Hg. (*a*) Machine milking rate. (*b*) Overall rate.

(2) *Machine milking rate and overall rate*

The results given in Fig. 3 and Tables 4*a* and 4*b* show that when the vacuum was increased from 11 to 20 in. Hg the machine milking rate increased from 1·03 to 1·62 lb./min., whereas the overall rate showed only a slight increase from 0·97 to 1·00 lb./min. Since the average rate of flow for cannulated milking at atmospheric pressure was low, the stripping of the other hind quarter of the same cow was delayed. The two treatments with vacuum at 16 in. for teat-cup milking (treatments Am and Cm) were therefore examined

separately for data relating to strippings. It can be seen from Table 4 that, while the machine milking rate for most quarters increased with increase in vacuum, in two cases the rates were lower for the 20 in. vacuum than for the 16 in. vacuum. For all quarters there was an increase in machine milking rate for increase in vacuum from 11 to 16 in. and from 11 to 20 in.; this indicates a real treatment effect. The lack of response in the overall rate of milking to increase in vacuum was due to the longer time necessary to remove the larger quantities of strippings left at the higher vacuum levels (see below).

Table 4*a*. *Machine milking rate* (*lb./min.*)

Cow	Hind quarter	Vacuum (in. Hg) 10·6	15·9	20·4	Mean	L. – R.
1	Left	0·71	1·08	1·31	0·97	0·14
	Right	0·71	0·88	1·11		
2	Left	1·30	1·88	2·50	1·70	0·38
	Right	1·27	1·70	1·37		
3	Left	1·06	1·20	1·11	1·61	−0·93
	Right	1·38	1·84	3·24		
4	Left	0·95	1·22*	1·22	1·09	0·12
	Right	0·85	1·07	1·14		
	Mean	1·03	1·36	1·62	1·34	

* One missing value estimated.

Table 4*b*. *Overall rate* (*lb./min.*)

Cow	Hind quarter	Vacuum (in. Hg) 10·6	15·9	20·4
1	Left	0·70	0·76	0·99
	Right	0·62	1·13	0·97
2	Left	1·26	1·35	1·03
	Right	1·08	1·21	1·08
3	Left	0·98	0·87	0·98
	Right	1·61	1·22	1·21
4	Left	0·74	0·80	0·83
	Right	0·75	0·85	0·88
	Mean	0·97	1·02	1·00

The overall rates for 15·9 in. vacuum given above and in Fig. 3 do not include those for which cannulated milking on the other hind quarter was carried out under atmospheric pressure.

(3) *Total milk yield*

In Fig. 4 and Tables 5*a* and 5*b* the total yields of the quarters for both teat-cup and cannula milking are given. There were no significant differences between the means, over all cows, of total yields for different levels of vacuum when the quarters were milked by teat cup. This indicates that variations in vacuum had little effect on the yield.

In comparing the figures for total yield from cannulated milking with those for teat-cup milking, it should be borne in mind that no stripping was done after cannulation. The inner lining of the teat tended to block the cannula towards the end of milking at the higher levels of vacuum, and the cannula was therefore withdrawn before milking was quite complete to avoid damage to the teat lining. For cannulation at atmospheric pressure, the mean value, over all cows, of the yield was the same as the mean value of the total yield for cows milked by teat cup at a vacuum of 20 in. Hg. With increase in vacuum, for cannulated

milking there was a slight decrease in milk yield, averaged over all cows, probably due to incomplete milking.

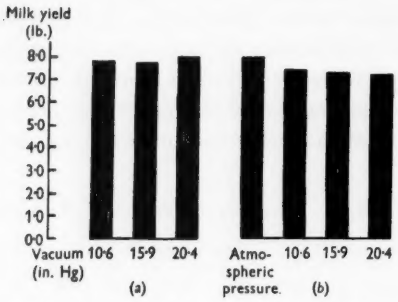

Fig. 4. The mean yields of milk of eight quarters when milked by teat cup at vacuums of 10·6, 15·9 and 20·4 in. Hg, and by cannula at atmospheric pressure and vacuums of 10·6, 15·9 and 20·4 in. Hg. (*a*) Milked by teat cup. (*b*) Milked by cannula.

Table 5. *Milk yields of quarters (lb.)*

(*a*) Milked by teat cup

		Vacuum (in. Hg)					
	Machine ...	10·6	15·9[x]	15·9[y]	20·4	Mean (±0·383)	L. – R.
Cow	Hind quarter						
1	Left	6·70	7·70	7·00	6·80	6·55	1·00
	Right	5·20	5·50	5·75	7·75		
2	Left	10·15	8·75	9·70	8·90	9·20	0·35
	Right	9·10	9·00	9·90	8·10		
3	Left	7·50	6·20	7·65	8·60	7·91	– 0·84
	Right	8·50	8·30	7·90	8·60		
4	Left	8·15	7·53*	8·00	7·30	7·58	0·32
	Right	7·20	7·25	7·30	7·95		
Mean	(±0·383)	7·81	7·53	7·90	8·00	7·81	—

* Missing value.

(*b*) Milked through cannula

			Vacuum (in. Hg)		
Cow	Hind quarter	Atmospheric pressure	10·6	15·9	20·4
1	Left	6·75	6·05	5·10	6·10
	Right	5·70	5·60	5·40	4·30
2	Left	9·70	9·20	10·20	8·25
	Right	9·00	9·60	8·05	9·50
3	Left	8·40	6·95	8·25	8·10
	Right	7·85	7·30	7·00	—
4	Left	7·60	7·20	6·80	7·45
	Right	8·90	7·75	7·80	6·80
Mean		8·00	7·46	7·33	7·21

x, y = yield when other hind quarter was cannulated at atmospheric pressure and 15·9 Hg respectively.

(4) *Yield of strippings*

The yield of machine strippings increased with increase in vacuum from a mean of 0·45 to 1·64 lb./quarter, an increase that was significant at the 3% level (see Table 6 and Fig. 5). It was found that though the initial rate of flow was very high when milking by teat cup at a vacuum of 20 in. Hg the rate soon fell and milk ceased to flow. If the vacuum was reduced to 11 in. Hg there was an immediate restart in milk flow and a

further small quantity was obtained. This result confirms the earlier finding of Smith & Petersen (3) and was probably due to greater teat-cup crawl at higher levels of vacuum (6). The fact that practically normal yields were obtained by cannula when no stripping or manipulation of the quarters was carried out suggests that the strippings obtained by teat-cup milking were due to mechanical rather than physiological factors.

Table 6. *Yield of machine strippings (lb.)*

		Vacuum (in. Hg)					
	Machine ...	10·6	15·9[x]	15·9[y]	20·4	Mean (±0·318)	L. – R.
Cow	Hind quarter						
1	Left	1·00	1·20	0·80	0·90	1·01	– 0·08
	Right	0·20	0·50	0·75	2·75		
2	Left	1·05	0·55	2·20	1·40	1·31	– 0·01
	Right	0·85	0·50	0·60	3·30		
3	Left	0·10	0·40	0·40	0·80	0·54	– 0·24
	Right	0·20	0·35	1·60	0·50		
4	Left	0·10	0·66*	0·45	1·80	0·82	– 0·14
	Right	0·10	0·10	0·65	1·70		
Mean (±0·318)		0·45	0·66	0·93	1·64	0·92	—

x, y. See footnote Table 5. * Missing value.

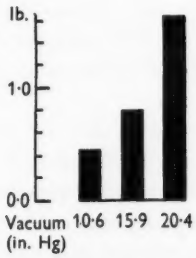

Fig. 5. The mean yields of machine strippings when the eight quarters were milked by teat cup at vacuums of 10·6, 15·9 and 20·4 in. Hg.

DISCUSSION

The experiment described above indicates that in machine milking, after 'let-down' has taken place, the maximum rate of flow is controlled by the size of the teat orifice and by the difference in pressure between the internal udder pressure and the milking machine vacuum. Where a constant vacuum is applied to the outside of the teat by the milking machine this pressure difference can only vary with changes in internal udder pressure. The work of Tgetgel (7) indicates that these internal changes are small compared with changes in milking machine vacuum.

The work reported here leaves little doubt that teat-orifice size is, in practice, a very important factor in controlling rate of milking and, in the eight quarters studied, the use of a cannula giving a constant bore for passage of milk resulted in very similar maximum rates of milking, although the same quarters when milked by teat cup had shown substantial differences in maximum rate of milking. These findings help to explain our failure in previous experiments to affect rate of flow by changes in milking routine (8, 9, 10), since the routines involved were unlikely to have much effect on the size of teat orifices.

Like the findings of Smith & Petersen (3) the experiment described above gives ample evidence of increased maximum rate of flow and some evidence of increase in average rate of milking, with increased vacuum. Large differences in milking rate between cows remained at the various levels of vacuum when milking was by teat cup.

The more rapid rise in the rate of milking with increasing vacuum in the case of teat-cup milking compared with milking through a cannula suggests that the teat orifice was stretched open at the higher vacuum levels. The fact that the faster milking quarters with large slack orifices apparently responded to a greater degree than the smaller orifices to increased vacuum, lends support to this suggestion.

Since the maximum rate of flow from a quarter which milked slowly by teat cup increased substantially when a suitable cannula was inserted, it is reasonable to suppose that the size of orifice normally remains a controlling factor in rate of flow for a substantial part of the milking period. For, as was noted above, once milk is 'let down' into the teats, the internal udder-pressure changes are too small to have a pronounced effect on rate of flow. It is, however, probable that changes in internal pressure may account for small differences in rate of flow. Thus, in testing the cannula with a reservoir of milk, it was found that a reduction in head of milk from 60 to 36 in., resulting in a comparatively small change in pressure difference, decreased the rate of flow from 2·73 to 2·60 lb./min., when the vacuum level in the machine was 16 in. Hg. We have often noted changes in rate of milking of this order in the same cow milked by teat cup at different stages of lactation.

It must be borne in mind that in the later stages of a milking when the teats are nearly empty, rate of drainage from the upper udder to the teats may become the factor controlling rate of flow.

The results of this experiment suggest that the use of higher vacuums than those commonly used in practice should not be encouraged. Apart from the danger of udder injury, a vacuum of 20 in. Hg may increase the quantity of strippings, although this may be influenced by the type of teat cup and liner used.

It was interesting to observe that normal milk yields were obtained when milking was carried out by a cannula at atmospheric pressure.

SUMMARY

1. A study was made of the milking of the two hind quarters of four cows by teat cup and by teat cannula at three levels of vacuum and by teat cannula at atmospheric pressure.

2. The eight quarters milked at significantly different maximum rates by teat cup but at nearly the same rate through a teat cannula, suggesting that the teat orifice is a very important factor controlling rate of milking.

3. The maximum rate of milking by both teat cup and teat cannula increased with increasing level of vacuum from 11 to 20 in. Hg. The rate of increase in the teat-cup milking was greater than in the cannula milking, suggesting that the teat orifice was stretched open at the higher levels of vacuum.

4. The average amount of strippings increased at the higher level of vacuum in teat-cup milking, due apparently to teat-cup crawl.

We wish to acknowledge the helpful advice of Dr R. Aschaffenburg regarding foam control.

APPENDIX I

Mean squares in the analysis of variance

(a) Milking by teat cup

	Peak rate of flow		Strippings		Total yield	
	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Between cows	3	2·9430**	3	0·8248	3	9·5456**
Between vacua:						
Linear	1	4·3623**	1	5·4867*	1	0·1177
Quadratic	1	0·0017	1	0·7235	1	0·3174
Between 15 in. vacua	—	—	1	0·2998	1	0·5513
Between days	6	0·0785*	6	0·4504	6	0·3095
Between quarters within cows	4	0·3155**	4	0·2986	4	0·3274
Cows *v.* vacua	9	0·0141	9	0·2316	9	0·3250
Residual	6	0·0133	5	0·8085	5	1·0818
Total	30		30		30	
Coefficient of variation		5·8%		97·5%		13·9%

(b) Cannulation

	Peak rate of flow	
	D.F.	M.S.
Between cows	3	0·0043
Between vacua:		
Linear	1	18·4220**
Quadratic	1	0·4185**
Remainder	1	0·1439
Between days	6	0·0277
Between quarters within cows	4	0·0523
Cows *v.* vacua	9	0·0019
Residual	5	0·0221
Total	30	
Coefficient of variation		6·6%

** Significant at 1% level. * Significant at 5% level.

The mean squares for days, quarters and cows *v.* vacua are the mean squares for each source of variation eliminating the other two. For peak rate of flow (machine milking) the 9 degrees of freedom for interactions include three for dummy interactions because of the duplications of the 15 in. vacuum treatment. The interaction mean square, however, was not significantly different from the error mean square even if calculated on 6 degrees of freedom. The mean square for all other sources of variation were tested against the residual mean squares.

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416. THE EFFECT OF BACTERIAL INFECTION ON THE MILK YIELD OF THE INDIVIDUAL QUARTERS OF THE COW'S UDDER

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REVIEW

The effect of mastitis on the yield and quality of the milk produced by individual quarters has been studied by a number of workers. Hastings & Beach (1, 2), Seelemann (3), Shaw & Beam (4), Horwood, Clark & Bryan (5), Ward & Castle (6), Edwards & Brownlee (7) and McDowall (8) all provide evidence that some quarters affected by mastitis give less milk than the corresponding non-infected quarters. Most of these workers relied on indirect tests for mastitis, while Hastings & Beach (1, 2) dealt with mastitis which was revealed by physical and chemical abnormalities in the milk of certain quarters and in which they could find no specific infection. These latter appear to be the only workers who referred to the yield of individual quarters before the onset of non-specific mastitis. Edwards & Brownlee (7) have also provided evidence of regeneration in the mammary gland during the dry period, following the treatment of clinically affected quarters near the end of the previous lactation.

McDowall (8) has shown that the total production of the abnormal quarters, diagnosed by the bromthymol blue test made on fore-milk samples, has a lower solids-not-fat percentage than that of the normal quarters. Determinations of solids-not-fat by most workers other than McDowall have been made on foremilk samples of 1–8 oz. or on the bulk milk of the whole udder. Neither of these sampling methods, however, gives a true picture of the effect of an infection on the solids-not-fat content of milk. This can only be assessed accurately by tests on the full yield of the individual quarter.

OBJECT

In order to obtain more information about progressive changes in the yield and composition of the milk of individual quarters, brought about by intramammary infections in the subclinical (latent or carrier) state, and also following successful treatment of clinical cases, a long-term investigation was started in March 1948. In addition to studies of *Streptococcus* infections, observations on *Staphylococcus* and *Micrococcus* infections were undertaken. These were of particular interest, since little is known of the effect of 'latent' infections caused by these organisms.

The investigation was carried out in a large tuberculin-tested herd free from contagious abortion and consisting of pedigree Shorthorn, Guernsey and Friesian cows. The cows were accustomed to machine milking with bucket-type machines operated at a vacuum of 14 in. mercury and a pulsation rate of 40 per min. They were washed immediately before milking with warm water, and were stripped at the end of milking by manipulation of the teat cups. Milk yields were recorded at every milking.

In the course of the investigation we have collected data from over one hundred cows,

some of them in two or three consecutive lactations. When studying the data it was evident that the infections produced a number of different and unpredictable results. Consequently, before analysing the data quantitatively, as will be done in a later communication, it was considered desirable to present some examples showing typical effects, and eleven case histories are given in this paper.

METHOD

The individual quarter bucket machine described by Hall (9) was used by experienced milkers at regular intervals on all cows in the herd. Non-infected cows were milked with this machine on four consecutive milkings once every 8 weeks, whilst infected cows were similarly milked every 4 weeks. On all other occasions the cows were milked in the normal manner as described above.

Sampling was carried out one afternoon at each of the monthly or 2-monthly sets of experimental milkings. The first ounce, taken from each quarter after careful disinfection of the teat orifice with hypochlorite, followed by drying, was used for the mastitis test and 4 oz. bulk samples from each quarter for chemical analysis. The yield given by each quarter was measured volumetrically at each milking when the quarter milker was used.

In addition to the foremilk samples taken when the individual quarter milker was used, whenever a test indicated that a change had occurred, two or three further samples were taken. Samples were also taken on drying off, during the dry period, and just before and after the animals calved.

Bacteriological methods

Milk samples were usually tested within 2 hr. of sampling, though occasionally they were tested after refrigeration overnight. About 0·05 ml. of each sample was plated on the surface of freshly poured plates of aesculin ox-blood agar using two large wire loops 6–7 mm. diameter. Samples from clinical cases were tested before and after dilution in skim milk. The plates were incubated for 2 days at 37° C. and the colonies examined and picked on to fresh blood plates and finally into litmus milk with chalk for further study. Streptococci were examined by serological and biochemical tests. The *Staphylococcus* haemolysin test of Munch-Petersen & Christie (10) for identifying group B streptococci was found very useful, whether using the *Staphylococcus* itself or the filtrate. Staphylococci were distinguished from micrococci by colony appearance, haemolysin production and by the slide coagulase test with human plasma.

Anaerobic culture and enrichment methods were adopted when advisable, but the results of enrichment tests alone were never accepted as diagnostic. Anaerobes were not found.

Whiteside test

The Whiteside test was made on all 1 oz. foremilk samples, and generally after refrigeration overnight. The method outlined by Murphy & Hanson (11) and Murphy (12) was used, except that from January 1949 two drops of alkali were used for both fresh and refrigerated samples. Cell counts using the Breed method were made occasionally to obtain additional information.

Electrical conductivity

Electrical conductivity determinations were made on the 1 oz. foremilk samples and on the 4 oz. full yield samples from each quarter. Only results of tests on the former samples are shown in the tables, for, in general, when the electrical conductivity of the 4 oz.

samples was regarded as abnormal the solids-not-fat percentage was also significantly altered.

An instrument known as a 'Rapid Abnormality Meter'* was used for the determinations, as this was readily available and was provided with a temperature compensator to give results at 18° C. The smallest divisions on the indicator scale represented 1×10^{-4} mho, and readings were made to the nearest 0·25 of a division.

Samples were raised to room temperature before testing, and each milk was tested at least twice, but frequently the cell was filled a third or fourth time before accepting the reading. The apparatus as supplied by the makers did not give the specific conductivity of a solution and had to have considerable adjustment. It was checked daily with a salt solution and a preserved milk sample, both of known specific conductivity, and only under such conditions gave reproducible results within $\pm 1 \times 10^{-4}$ mho.

Differences in the conductivity between quarter samples were on a number of occasions checked by a chloride determination and the differences confirmed.

Solids-not-fat

The estimation of the percentage solids-not-fat was determined by the difference between the total solids estimated gravimetrically and fat by the Gerber test. The solids-not-fat determinations for each quarter were compared. When the percentage in the milk of one quarter was 0·30 less than that of any other quarter, it was regarded as indicative of abnormality (13). This method of comparing the tests on one quarter with those of the other three quarters of the same udder has been used because it is now well recognized as a much more sensitive indicator than the previous method of comparing the results for each quarter with an arbitrary standard (VanLandingham, Weakley, Moore & Henderson (14); Rowland (15)).

The determinations of the fat contents of the bulk milks carried out by the Gerber method have not been included in the tables or in the discussions of the results. They have been excluded because estimates of fat production based on the results of single determinations are known to be very unreliable.

RESULTS

Before describing the eleven case histories of infected cows, it should be recorded that the individual quarter milking machine when used on uninfected udders gave results which in general agree with those of Turner (16) and Matthews, Swett & Graves (17). On average the hind quarters of non-infected udders each give slightly less than 30% of the total production of the udder and each front quarter slightly more than 20%. Individual cows show a variation from this pattern, though it is not usual for the differences in the proportionate yields of the two front or the two hind quarters of an udder that has not been infected to show marked differences. The mean of the differences between the proportionate yields (expressed as a percentage of the total production) of the front quarters of thirty non-infected cows at the first test after calving was 1·30 and between the hind quarters 1·55. Normally, the proportionate yields of these quarters do not show marked changes with advance in lactation, though there is some evidence that forequarters dry off at a slightly quicker rate than hind quarters.

* Supplied by Industrial and Scientific Instruments Ltd., 133 Euston Road, London, N.W. 1.

The particulars concerning the eleven infected cows are given in the subsequent eleven tables and on the pages facing each table.

The condensed tables, each covering a period of about one lactation, show the individual quarter yields and the results of the bacteriological, Whiteside and conductivity tests on the fore-milk samples and the solids-not-fat tests on the full yield samples, together with notes on the clinical state of the udders and the treatments received. No attempt has been made to show the proportion of cows which responded in any particular way.

The infections in the eleven cows described comprise eight caused by streptococci, nine by staphylococci and five by micrococci. They may be roughly classified in four main groups according to their effect on the relative milk yield of the quarter and on the results of the tests for abnormality.

(1) Subclinical infections having no measurable effect on the relative milk yield of the quarter and causing little disturbance to the gland as determined by the Whiteside and conductivity tests on foremilk samples. These are depicted by the *Micrococcus* infections shown in Tables 3, 4, 9 and 10 and the *Streptococcus dysgalactiae* infection shown in Table 4.

(2) Subclinical infections having no measurable effect on milk yield but causing irritation of the gland as determined by indirect tests on foremilk samples. These results occurred with the *Str. agalactiae* infections shown in Table 3, the *Str. dysgalactiae* infection, Table 4, and the *Staphylococcus* infections, Table 5.

(3) Subclinical infections causing a progressive drop in the yield of the affected quarter and sufficient irritation to produce abnormalities in both the foremilk and that of the full yield of the quarter as measured by the indirect tests described. Examples are the *Str. uberis* and *Staphylococcus* infections shown in Table 2 and the *Staphylococcus* infections shown in Tables 1 and 9.

(4) Infections causing obvious clinical symptoms and an abrupt fall in milk yield often greater than that caused by subclinical infections of long duration. Examples are the *Str. agalactiae* infections shown in Tables 6 and 7 and the *Staphylococcus* infections shown in Tables 8, 10 and 11.

Most of the clinical cases were treated with penicillin within 48 hr. of the first observed symptoms. After the infection had apparently been removed, the indirect tests made on the foremilk, and often those on the full yield of the quarter, remained positive for some weeks or months (Tables 7, 8 and 10), and the relative yield showed little increase until the next lactation (Table 11).

The original data have been condensed in the following tables to conserve space and to avoid repetition.

The key to the tables will be found on p. 154.

Table 1. *Campion* 17

An example of a subclinical *Staphylococcus* infection which caused a reduction in the yield of the affected quarter and a significant change in the composition of the milk.

Data collected in the first lactation are shown in the table.

This cow started her first lactation free from udder infection and with almost normal yields from each quarter. In the third or fourth month of this lactation the R.F. quarter became infected with haemolytic staphylococci which were found at every test and persisted into the second lactation. Clinical symptoms were not observed on any occasion in the first lactation.

There was a progressive fall in the proportionate yield of the quarter from 23% of the total daily production in November to 12% in June, when the quarter had been infected for about 5 months.

The indirect tests for abnormal milk were nearly always positive for the R.F. quarter once the infection was found but the reactions were not strong, though the electrical conductivity tended to rise in later lactation. The indirect tests for the other quarters were always negative, except for a few positives in late lactation. There was a significant fall in the solids-not-fat content of the milk compared with that of the other quarters, a depression which persisted to the end of the lactation (i.e. for 4 months). The electrical conductivity readings made on the full yield of the R.F. quarter were also abnormal while the infection was present.

This staphylococcal infection persisted throughout the dry period at the end of the first lactation and became clinical early in the second lactation.

Table 1. *The first lactation of Campion* 17 (*Shorthorn*)

Individual quarter yields (lb.) (%)				Daily milk yield		Bacteriological results				Whiteside test				Conductivity difference				Solids-not-fat %			
R.F.	L.F.	R.H.	L.H.	(lb.)	Date	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.
23·3	21·8	27·5	27·4		1948																
					10. 10	32*m*	10*m*	4*m*	22*mx*	1	—	—	—	4	—	2	—	**1st calving**			
					11. 10	10*m*	4*m*	9*m*	61*mx*	1	—	—	—	—	3	—	—				
				25·8	19. 10	4*m*	3*m*	10*m*	—	—	—	—	—	—	—	—	—	9·51	9·44	9·58	9·63
				30·1	**1. 11**	8*m*	—	4*m*	—	—	—	—	—	—	—	—	—	8·89	8·91	9·90	8·96
23·5	21·7	27·9	26·9																		
				28·4	**15. 11**	5*m*	7*m*	6*m*	—	—	—	—	—	—	—	—	—	9·14	9·06	9·08	9·15
					13. 12	35*mx*	3*m*	1*m*	2*m*	—	—	—	—	—	—	—	—				
19·5	21·7	29·8	28·9		1949																
				23·6	**7. 2**	**200 S**	—	—	1*m*	1	—	—	—	—	—	—	—	8·98	9·12	9·18	9·15
					8. 2	**250 S**	4*mx*	7*mx*	—	—	—	—	—	2	—	—	—				
					10. 2	**250 S**	3*m*	20*mx*	3*m*	—	—	—	—	3	—	—	—				
					11. 2	**200 S**	2*m*	8*mx*	7*mx*	1	—	—	—								
17·1	22·4	30·9	29·6		17. 2	**200 S**	4*mx*	2 *C*	3*m*	1	—	—	—	—	—	—	—	**8·86**	9·13	9·26	9·17
				22·3	**16. 3**	**200 S**	2*m*	2*m*	2*m*	1	—	—	—	4	—	—	—	**8·97**	9·11	9·34	9·20
					25. 3	**200 S**	—	6*m*	3*m*	2	—	—	—	2	—	—	—				
					29. 4	**120 S**	—	1*m*	2*m*	1	—	—	—	2	—	—	—				
16·4	22·4	32·5	29·7	21·1	**11. 5**	**300 S**	—	1*m*	—	1	1	—	—	6	6	—	—	**9·40**	9·76	9·75	9·82
					26. 5	**250 S**	3*m*	—	—	1	1	—	—	—	4	—	—				
12·4	21·5	34·8	31·3	14·8	**28. 6**	**150 S**	3*m*	—	—	2	—	—	—	12	5	—	6	**9·08**	9·54	9·78	9·74
					3. 8	**∞ S**	3*mx*	—	3*m*	3	3	1	2	12	13	—	7	**Dry 8. 8. 49**			

Table 2. *Campion* 15

An example of subclinical *Streptococcus uberis* and *Staphylococcus* infections which caused reductions in the milk yields of the affected quarters and small but definite decreases in the percentage of solids-not-fat.

Data collected in the third lactation are shown in the table.

In her first lactation this animal had a haemolytic *Staphylococcus* infection in her L.F. quarter. She was free from infection in her second lactation but developed a *Str. uberis* infection in her L.F. quarter during the subsequent dry period. In the third lactation which is illustrated in the table this *Str. uberis* infection persisted until the end of the lactation, together with a *Staphylococcus* infection in her L.H. quarter which started in the fifth month. In neither of these quarters were clinical symptoms found during the period under review.

Both the infections were associated with a fall in the relative milk yields of the quarters. The proportion of the daily yield contributed by the L.F. quarter was about 17% at the start of the lactation and about 9% 8 months later, while the proportion contributed by the L.H. quarter fell from 35 to 28% in 4 months.

At the start of the third lactation the yields of the front and hind quarters were uneven, and it is possible that the lower yield of the L.F. quarter was caused by the infection in the dry period. There is no explanation of the large difference in the yields of the R.H. and L.H. quarters, apart from the possibility of a temporary infection in the R.H. quarter at calving, which was not diagnosed. It is of some interest to note that the difference between the relative yields of the two apparently normal quarters widened considerably as the lactation advanced. The ratio of the yield of the R.F. to the R.H. fell consistently from 0·89 to 0·47 in 8 months. Unless coincidental there appears to be no explanation for this other than that compensation occurred in the R.H. quarter. This explanation is considered likely, for while the total yield of the cow was falling the actual yield of the R.H. quarter was practically unchanged and that of the R.F. decreased as expected.

The solids-not-fat percentage showed a significant drop in the milk of the L.F. quarter after the second month of lactation, but the solids-not-fat percentage of the milk of the L.H. quarter was not significantly low until the eighth month of lactation.

The indirect tests on the foremilk of the infected quarters were nearly always positive once the infections were established. The reason why the foremilk of the R.F. quarter was positive to the Whiteside and conductivity tests in the seventh and eighth months of lactation is not clear, but such occurrences are common in low-yielding quarters near the end of a lactation.

Table 2. *The third lactation of Campion* 15 (*Shorthorn*)

Individual quarter yields (lb.) (%)				Daily milk yield (lb.)	Date	Bacteriological results				Whiteside test				Conductivity difference				Solids-not-fat %			
R.F.	L.F.	R.H.	L.H.			R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.
					1948																
					12. 8	300*m*	**200 III**	300*m*	2*m*												
					25. 9	70*m*	**900 III**	32*m*	—									**Dry period**			
					27. 9	1*m*	**8 III**	1*m*	2*m*	—	2	—	2	—	—	—	—	**3rd calving**			
					29. 9	100*m*	**∞ III**	60*m*	3*m*	—	3	—	—	—	3	—	2				
					29. 9	1*m*	**∞ III**	—	1*m*	—	1	—	—	—	3	—	—				
21·2	16·8	27·2	34·8	35·8	**4. 10**	2*m*	**800 III**	2*m*	—	—	1	—	—	—	5	—	—	9·05	9·12	9·13	9·09
				35·2	2. 11	3*m*	**500 III**	10*m*	1*m*	—	2	—	—	—	5	—	—				
21·2	12·2	31·7	35·0	29·7	7. 12	56*m*	**800 III**	11*m*	7*m*	—	1	—	—	—	—	—	—				
					19. 12	2*m*	**800 III**	14*m*	80*m*	—	2	—	—	—	2	—	—	9·05	**8·84**	9·11	9·21
					1949																
				25·9	**27. 1**	12*m*	**700 III**	84*m*	**200 S**	—	4	—	—	—	6	—	—	8·90	**8·59**	9·00	8·72
					21. 2	6*mx*	**∞ III**	20*m*	**250 S**	—	4	—	—	—	19	—	—				
					30. 3	3*m*	**∞ III**	3*m*	**200 S**	—	2	—	1	—	14	—	2				
21·3	10·2	38·6	30·0	21·8	**27. 4**	15*mx*	**300 III**	15*mx*	**200 S**	3	2	1	3	3	25	—	9	8·69	**8·46**	8·79	8·56
20·1	9·3	42·3	28·3	15·6	**25. 5**	1*m*	**200 III**	8*m*	**250 S**	1	3	—	2	13	27	—	26	**8·39**	8·55	8·80	**8·26**

Table 3. *Medlar*

An example of some subclinical *Streptococcus agalactiae* infections which have little or no effect on the relative yield of the affected quarters.

Particulars of the first lactation are shown in the table.

This animal started her first lactation with almost normal quarter yields and with *Micrococcus* infections in three quarters. The R.H. quarter became infected with haemolytic staphylococci in the fourth month of the first lactation following an injury to the tip of the teat, and in the fifth month, both the R.H. and R.F. quarters were infected with *Str. agalactiae*.

These infections remained for the rest of the lactation without producing any clinical symptoms. The infection in the R.F. quarter did not cause any reduction in yield, though the production of the R.H. quarter may have been slightly affected. The changes in the relative hind-quarter yields are difficult to interpret. It appears that the L.H. quarter increased relative to the other three quarters. On the other hand, it can be argued that the R.H. quarter decreased because of its infection and the front quarters also decreased at a similar rate. There is some evidence that front quarters dry off more quickly than hind quarters, even with non-infected cows. In either case it is evident that the mastitis had only a minor effect.

The infections had no measurable effect on the solids-not-fat percentage of the milk, but the indirect tests made on the foremilks were nearly always positive except for the R.H. quarter which was only occasionally positive to the conductivity test.

The *Micrococcus* infection in the L.H. quarter persisted throughout the lactation but apparently caused little disturbance to the gland.

Table 3. *The first lactation of Medlar (Guernsey)*

Individual quarter yields (lb.) (%) R.F.	L.F.	R.H.	L.H.	Daily milk yield (lb.)	Date	Bacteriological results R.F.	L.F.	R.H.	L.H.	Whiteside test R.F.	L.F.	R.H.	L.H.	Conductivity difference R.F.	L.F.	R.H.	L.H.	Solids-not-fat % R.F.	L.F.	R.H.	L.H.
21·5	21·9	29·7	26·9		1948																
					11. 12	350*m*	3*m*	250*m*	200*m*	1	—	1	1					**1st calving**			
				23·9	13. 12	80*m*	4*m*	600*m*	∞ *m*	1	—	1	1								
					15. 12	80*m*	—	700*m*	∞ *m*	—	—	—	—	—	—	—	2				
				34·3	**30. 12**	3*m*	2*m*	160*m*	200*m*	—	—	—	—	—	—	—	—	8·92	8·87	9·09	8·95
					1949																
					13. 1	6*m*	—	150*m*	350*m*	—	—	—	—	—	—	—	—				
21·6	22·1	28·4	27·9		7. 2	—	—	∞ *m*	150*m*	—	—	—	—	—	—	—	—	9·11	9·14	9·14	9·01
				31·6	**21. 2**	6*mx*	1*m*	2*m*	100*m*	—	—	—	—	—	—	—	—				
22·1	22·4	26·5	29·0																		
				28·4	**28. 3**	—	1*m*	**350 S**	250*m*	—	—	4	—	—	—	4	—	8·67	8·75	8·65	8·71
					4. 4	—	1*m*	∞ **S**	400*m*	—	—	2									
21·1	21·9	27·7	29·4		28. 4	**300 I**	—	∞ **S** ∞ **I**	300*m*	3	—	1	2								
					4. 5	∞ **I**	—	**120 S** **150 I**	150*m*	2	—	1	—	3	—	—	—	9·36	9·12	9·26	9·26
				27·1	**30. 5**	**20 I**	—	**200 S** **200 I**	120*m*	3	1	3	1	9	—	5	—	9·48	9·35	9·56	9·46
					8. 6	**70 I**	—	**150 S** **250 I**	200*m*	1	—	1	—	6	—	—	—				
19·7	21·3	27·6	31·4																		
				24·0	4. 7	**50 I**	—	**150 S** **60 I**	160*m*	2	1	2	1	4	—	—	—	9·13	9·09	9·24	9·14
				19·3	**23. 8**	**300 I**	2*n*	**200 S** **250 I**	250*m*	2	1	2	1	3	—	2	—	9·40	9·29	9·42	9·40
					29. 8	**120 I**	—	**60 S** ∞ **I**	∞ *m*												
19·5	20·8	26·7	33·0																		
					26. 9	**150 I**	—	**100 S** **200 I**	250*m*	4	1	4	1	3	—	—	—				
				15·3	**25. 10**	∞ **I**	—	**120 S** **300 I**	300*m*	1	1	4	1	—	—	—	—	9·26	**9·03**	9·28	9·33
					8. 11	**200 I**	—	**250 S** ∞ **I**	120*m*	2	—	2	—	2	—	2	—				

Table 4. *Flora* 63

An example of a subclinical *Streptococcus dysgalactiae* infection of long duration unaccompanied by a reduction in the proportionate yield of the affected quarter.

Data collected in the sixth lactation are shown in the table.

This animal started her sixth lactation with quarter yields practically normal, and in spite of a persistent *Str. dysgalactiae* infection she also completed her lactation with normal quarter yields.

In her fourth lactation Flora 63 was clinically affected in both forequarters by a *Str. agalactiae* infection. During the fifth lactation the R.F. quarter became infected with *Str. dysgalactiae*, the infection persisting throughout the lactation, the following dry period and also the sixth lactation as illustrated in the table. The infection had no effect on the relative milk yield, in spite of the fact that in the fifth month of the sixth lactation the milk from this quarter contained a few clots and was strongly positive to the Whiteside and conductivity test. The solids-not-fat percentage was significantly reduced at this time but apparently only for a short period. It is unusual for the solids-not-fat to be significantly affected unless the quarter has also shown a reduction in milk yield.

It is also of interest to note that except on the occasion when clots were present in the milk the conductivity test was nearly always negative or barely positive, and that the Whiteside test was occasionally negative, though the streptococci were regularly recovered and the teat orifice was healthy. Clots were constantly present in the secretion of the R.F. quarter during the last 5 days of the sixth lactation, but the quarter was free from clinical symptoms at the start of the seventh lactation.

During the dry period after the end of the sixth lactation two additional quarters became infected with *Str. dysgalactiae*. The proportionate yields of the quarters were normal after the seventh calving, except for a slight reduction in yield caused by one of the new infections. It is interesting to record that the R.F. quarter of this cow was infected for over 2 years without showing any measurable effect on milk yield.

This example is given to illustrate that an organism commonly associated with acute mastitis can persist in some udders without producing severe symptoms.

Table 4. *The sixth lactation of Flora* 63 (*Shorthorn*)

Individual quarter yields (lb.) (%)				Daily milk yield (lb.)	Date	Bacteriological results				Whiteside test				Conductivity difference				Solids-not-fat %			
R.F.	L.F.	R.H.	L.H.			R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.
18·9	18·5	29·9	32·7		1948																
					20. 2	∞ II	—	—	—									**Dry period**			
					29. 4	∞ II	7*m*	400*m*	—	1	—	—	—					**6th calving**			
					7. 5	**400 II**	—	80*m*	—	1	—	—	—								
				54·0	**12. 5**	**400 II**	—	150*m*	—	1	—	—	—	2	—	—	—	9·24	9·24	9·42	9·16
					7. 6	**150 II**	40*m*	100*m*	40*m*	1	—	—	—	—	—	—	—				
19·3	17·4	29·7	33·6		10. 6	**200 II**	5*m*	75*m*	50*m*	1	—	—	—	—	—	—	—				
				50·0	**5.7**	**70 II** 25*m*	75*m*	50*m*	11*m*	1	—	—	—	—	—	—	—	8·50	8·54	8·73	8·63
18·8	17·4	30·8	33·0	44·8	**3. 8**	**150 II**	100*m*	50*m*	10*m*	1	—	—	—	2	—	—	—	**8·32**	8·63	8·67	8·66
					30. 8	**150 II** 150*m*	55*m*	120*m* 2 *S*	10*m*	—	—	—	—	—	—	—	—				
17·6	17·8	31·8	32·8	44·6	**29. 9**	**120 II**	250*m*	230*m*	200*m*	4	—	—	—	6	—	—	—	**8·46**	8·99	9·23	9·16
					8. 10	**250 II** 150*m*	150*m*	50*m*	5*m*	—	—	—	—	2	—	—	—				
19·6	19·6	27·3	33·5	35·1	**25. 10**	**50 II** 200*m*	100*m*	80*m*	27*m*	—	—	—	—	—	—	—	—	9·06	9·18	9·11	9·24
				9·5	30. 12	**500 II** 200*m*	400*m*	150*m*	600*m*	4	3	3	3	9	—	—	—	**9·05** **Dry 9. 1. 49**	9·48	9·54	9·25

Table 5. *Campion* 12

An example of subclinical *Staphylococcus* infections which did not cause a proportionate reduction in the yields of the infected quarters.

Data collected during the fourth lactation are shown in the table.

This cow, free from udder infections in her second lactation, developed haemolytic *Staphylococcus* infections in her forequarters during the third lactation. The infections persisted throughout the dry period and fourth lactation (see table) without any obvious clinical symptoms being observed.

The table shows that the relative yields of all quarters remained remarkably constant, though the staphylococci were always recovered from the forequarters. The direct tests on the foremilk of these two quarters were nearly always positive, while the solids-not-fat percentage of the total yield from each quarter remained unaffected. During a part of July and August the solids-not-fat percentage of the milk of all quarters was between 8·0 and 8·5.

In the dry period before the fifth lactation (not shown) the L.H. quarter became infected with *Streptococcus uberis* and haemolytic staphylococci. The *Str. uberis* infection did not persist, and on calving the quarter had a clinical *Staphylococcus* infection which was treated with penicillin. The subsequent quarter milking revealed a drop in the relative production of the L.H. from 36% of the total production at the end of the fourth lactation to 11% at the beginning of the fifth.

The L.F. quarter lost its *Staphylococcus* infection just before the start of the fifth lactation, while the infection persisted in the R.F. quarter. Nevertheless, the proportionate yields of the front quarters at the start of the fifth lactation were virtually the same, being 23·5 and 23·4%.

Table 5. *The fourth lactation of Campion* 12 (*Shorthorn*)

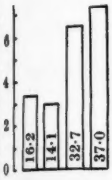
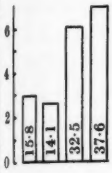
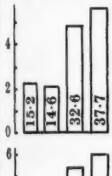
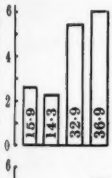
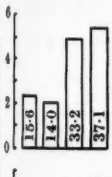
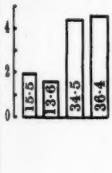
Individual quarter yields (lb.) (%)	Daily milk yield (lb.)	Date	Bacteriological results				Whiteside test				Conductivity difference				Solids-not-fat %			
			R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.
 R.F. 16·2 L.F. 14·1 R.H. 32·7 L.H. 37·0	38·9	1948 19. 3 26. 3 1. 4 5. 4 **31. 4** 1. 6	**400 S** — **20 S** **150 S** **250 S** **250 S**	**150 S** — **37 S** **150 S** **50 S** **50 S**	**300 III** — — — 1*m* —	— — — — 1*m* —	2	1	—	—	4	—	—	—	8·92	8·91	9·00	9·06
 R.F. 15·8 L.F. 14·1 R.H. 32·5 L.H. 37·6	37·2	**29. 6**	**20 S** 40*m*	**50 S**	2*m*	—	2	1	—	—	9	4	—	—	8·57	8·75	8·79	8·78
 R.F. 15·2 L.F. 14·6 R.H. 32·6 L.H. 37·7	29·7	**26. 7**	**20 S** 50*m*	**50 S**	—	—	1	—	—	—	2	—	—	—	8·25	8·50	8·43	8·44
 R.F. 16·9 L.F. 14·3 R.H. 32·9 L.H. 36·9	32·5	**23. 8**	**50 S** 150*m*	**150 S**	2*m*	2*m*	2	1	—	—	14	3	4	—	8·25	8·38	8·32	8·34
 R.F. 15·6 L.F. 14·0 R.H. 33·2 L.H. 37·1	29·5	**23. 9**	**350 S**	**300 S**	1*S* 17*m*	—	3	2	1	—	23	5	4	—	8·75	8·80	8·93	8·94
 R.F. 15·5 L.F. 13·6 R.H. 34·5 L.H. 36·4	25·5	**19. 10** 15. 11	**400 S** **300 S**	**200 S** **500 S**	10*m* —	1*m* —	2 1	1 —	1 —	— —	25 2	5 —	5 —	— —	8·92 **Dry 2. 1. 49**	8·83	9·06	9·03

Table 6. *Pandora*

The change in the relative yield of a quarter, clinically affected after an injury to the teat and the effect of a second clinical condition in the same quarter.

The history of the first lactation is shown in the table.

This animal started her first lactation free from udder infections and with normal quarter yields. A month later the tip of her L.H. teat was slightly injured, and this was followed in a few days by a clinical attack. Treatment resulted in a clinical cure but not a bacteriological cure, as the sore at the orifice persisted longer than the treatment. The original infection, which appeared to be a mixed haemolytic *Staphylococcus* and *Streptococcus dysgalactiae* infection, became a mixed *Staphylococcus* and *Str. agalactiae* infection, and finally, 4 months later, a pure *Streptococcus* infection. About 5 months after the first clinical condition the quarter was again clinical, and penicillin treatment produced a complete cure.

Though the yields of the hind quarters were equal before the onset of the infection, the first clinical attack reduced the yield of the L.H. to 57 % of the R.H. Three months later this had been reduced to 40 % of the R.H. by the subclinical infection, while the subsequent clinical attack did not cause any further fall in yield. It is interesting to record that, in this case, the subclinical attack caused a much more serious decrease in yield than the following clinical outbreak.

The solids-not-fat percentage in the milk of the infected quarter remained significantly low for the duration of the infection and the indirect tests were constantly positive.

The reason for the abnormal conductivity and Whiteside tests for the milk of the R.F. quarter after the fifth month of lactation is not known. Such findings are not uncommon.

Table 6. *The first lactation of Pandora (Guernsey)*

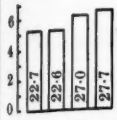
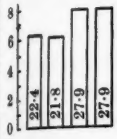
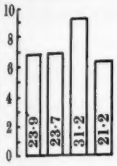
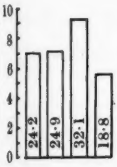
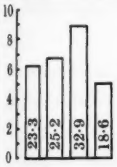
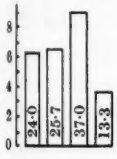
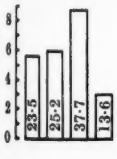
Individual quarter yields (lb.) (%) R.F.	L.F.	R.H.	L.H.	Daily milk yield (lb.)	Date	Bacteriological results R.F.	L.F.	R.H.	L.H.	Whiteside test R.F.	L.F.	R.H.	L.H.	Conductivity difference R.F.	L.F.	R.H.	L.H.	Solids-not-fat % R.F.	L.F.	R.H.	L.H.
 22·7	22·6	27·0	27·7		1948																
					26. 12													**1st calving**			
					27. 12	5*m*	6*m*	—	3*m*												
				23·3	**30.12**	2*m*	—	—	1*m*	1	1	—	—	—	—	—	—	9·36	9·31	9·09	9·07
 22·4	21·8	27·9	27·9		1949																
				27·8	**7.1**	12*mx*	6*m*	16*mx*	—	—	—	—	—	—	—	—	—	9·15	8·97	9·14	9·12
					13. 1	—	20*m*	—	6*mx*	—	—	—	—								
 23·9	23·7	31·2	21·2		26. 1	10*mx*	6*mx*	7*mx*	**30 S** 250 II									**L.H. teat injured 21. 1. 49**			
					27. 1	2*m*	1*m*	4 *C*	**∞ S**	—	—	—	4	—	—	—	9				
									←									**L.H. Pen.** (5×100,000)			
				29·0	**2.2**	6*mx*	3*m*	11 *S*	**300 S**	—	—	—	2	—	—	—	4	8·97	9·00	9·08	9·16
					7. 2	—	—	1*m*	**7 S** 200 I	—	—	—	4	—	—	—	7				
 24·2	24·9	32·1	18·8		14. 2	4*m*	—	—	**150 S** ∞ I	—	—	—	1	—	—	—	3				
				29·0	**28. 2**	—	—	2*m*	**50 S** 400 I	—	—	—	2	—	—	—	6	8·97	9·06	9·13	**8·70**
					7. 3	—	—	3*m*	**300 S** ∞ I	1	—	—	1	—	—	—	4				
 23·3	25·2	32·9	18·6		21. 3	4*m*	6*mx*	1 *C*	**600 S** 300 I	—	1	—	3	—	—	—	6				
				26·6	**11. 4**	1*m*	4*m*	30*mx*	**100 S** 200 I	—	—	—	2	—	—	—	6	9·15	9·42	9·18	**8·66**
 24·0	25·7	37·0	13·3	26·1	**16. 5**	—	—	—	∞ I	1	1	—	3	3	2	—	8	9·79	9·64	9·85	**8·28**
					15. 6	—	—	2*m*	**250 I**	—	—	—	2	2	—	—	7	9·34	9·46	9·36	**8·96**
					17. 6	—	—	6*m*	**20 I**	1	1	—	4	5	—	—	13				
									←									**L.H. Pen.** (2×100,000)			
 23·5	25·2	37·7	13·6		24. 6	—	1*m*	—	1*m*	—	—	—	1	—	—	—	5				
				23·4	**6.7**	—	—	2*m*	2*m*	1	—	—	—	3	—	—	2	9·04	9·12	9·22	8·95
					8. 7	—	—	—	2*m*	1	—	—	—	4	—	—	—	**Dry 17. 10. 49**			

Table 7. *Niobe*

An example of the marked effect that some clinical *Streptococcus agalactiae* infections have on the relative yields of the affected quarters.

Particulars of the fifth lactation of this cow are shown in the table.

The tip of the teat of the L.H. quarter of this animal was damaged when trodden on early in her fourth lactation and became infected with *Corynebacterium pyogenes*, *Str. agalactiae* and staphylococci. By means of a silver bougie, penicillin and acriflavine ointment, the quarter was eventually saved and the infection eliminated. The R.F. and L.H. quarters were found to be infected with *Str. agalactiae* at the start of the fifth lactation (see table), while the L.F. and R.H. quarters were possibly infected with staphylococci. Within a few weeks the staphylococci in the L.F. were replaced by *Str. agalactiae*.

The R.F. and L.F. quarters became clinical in the fourth month of lactation, both quarters being swollen and inflamed whilst the secretion consisted of fine yellow clots. They were readily cured, however, with a small infusion of penicillin after being clinical for 48 hr. The milk yield of both quarters was found to have been greatly reduced, when measured 24 hr. after the first clinical symptoms were noticed, and there was only a slight further drop before treatment was completed. Measurements made in July and again in August (not shown) showed a slight increase in the yield of the front quarters.

The solids-not-fat content of the milk of both the front quarters remained depressed after the streptococci had been eliminated, and it continued significantly low in the milk of the L.F. quarter for another 2 months or more, though the *Streptococcus* was not recovered from any of the thirteen samples taken.

The effect of the subclinical infection in the R.H. and L.H. quarters is hard to estimate. While both were positive to the Whiteside test neither appeared to give milk of abnormal solids-not-fat percentage or conductivity. Since these latter tests are based on the assumption that the quarter with the highest solids-not-fat percentage and the lowest conductivity reading is the 'normal', they are, as is well known, not reliable tests when all four quarters are infected. It is quite probable, since the conductivity of the R.H. and L.H. was high in comparison with a non-infected cow, that these subclinical infections were reducing yield. This assumption may help to explain the changes in the relative yields of the two hind quarters as lactation progressed, the staphylococcal infection in the R.H. quarter having a greater effect than the mixed infection in the L.H. quarter.

Table 7. *The fifth lactation of Niobe (Guernsey)*

Individual quarter yields (lb.) (%)				Daily milk yield		Bacteriological results				Whiteside test				Conductivity difference				Solids-not-fat %			
R.F.	L.F.	R.H.	L.H.	(lb.)	Date	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.
					1948																
					7. 1																
					10. 1	**150 I**	**15 S** 5*m*	**60 S**	**400 I** **2 S**									**5th calving**			
16·5	23·0	39·8	21·7	31·7	19. 1	**50 I**	**20 S**	—	**30 I**												
				31·9	20. 2	**150 I**	**12 I**	1*m*	**100 I**												
				34·8	3. 3	**400 I**	**10 I**	1*m*	**100 I**												
				24·2	**23. 3**																
					16. 4	**100 I**	**300 I**	**1 S**	**300 I**	1	1	—	1								
8·7	10·0	48·2	33·1		26. 4	**∞I**	**∞I**	**4 S**	**250 I** **100 S**	4	4	2	2								
				18·0	**27. 4**	**∞I**	**∞I**	**60 S**	**300 I** **200 S**	4	4	2	1	32	32	—	—				
						←	←											**R.F., L.F. Pen.** (2 × 20,000)			
7·5	6·6	49·9	36·0	18·9	**10. 5**	**2 S**	—	**75 S**	**150 I** **100 S**	2	2	1	1	6	7	—	—	**8·14**	**7·98**	8·78	8·69
					14. 6	—	—	—	**250 I** **150 S**	2	3	2	2	—	5	—	—	8·66	**8·52**	8·85	8·86
10·3	8·8	45·3	35·6		15. 6	—	—	—	**400 I** **10 S**	1	1	1	1	2	—	—	—				
				17·8	5. 7	—	2*m*	**3 S**	**50 I** **25 S**	1	1	2	2	4	3	—	—	8·65	**8·44**	8·81	8·62
					3. 8	—	1*S*	**100 S**	**200 I**												
					12. 8	—	12*m*	**200 S**	**200 I**												

Table 8. *Pippin* 12

An example of a drop in milk yield in a quarter clinically affected by a *Staphylococcus* infection in mid-lactation and of a further drop in yield following an apparent cure.

The history of the first lactation is shown in the table.

Like Campion 17, this animal started her first lactation free from udder infection and with very nearly normal yields from each quarter. In the third month of lactation the R.H. quarter became swollen and clots were found in the foremilk at one or two milkings though the milk was not discoloured. The swelling was less evident 24 hr. later, and the infection quickly responded to penicillin treatment.

Although penicillin treatment was given 48 hr. after clinical symptoms were first noticed, the milk yield of the affected quarter had already been depressed. Though the haemolytic staphylococci had apparently been eliminated the yield of the quarter continued to decline by a small, though definite, amount relative to the yields of the L.H. quarter and also to that of the combined yield of the front quarters. The reason for this slight but progressive decline in relation to the yield of the front quarters is not clear. Although there is the possibility that the quarter was still infected this is unlikely, because no staphylococci were isolated from subsequent milk samples or from the secretion in the dry period. Furthermore, there was a complete recovery in the yield of this quarter at the start of the next lactation when each hind quarter was giving 27·4% of the daily yield. A possible explanation of this persistent decrease in yield after an apparent cure is that the duct system was damaged during the clinical attack. This damage might prevent the removal of the normal quantity of milk from the upper udder, and in this way increase the rate of drying off.

Following treatment of the R.H. quarter, the indirect tests for abnormal secretion remained positive for three months, though the solids-not-fat percentage in May was significantly low only when expressed on a 'fat-free serum' basis.

Table 8. *The first lactation of Pippin* 12 (*Shorthorn*)

Individual quarter yields (lb.) (%)				Daily milk yield (lb.)	Date	Bacteriological results				Whiteside test				Conductivity difference				Solids-not-fat %			
R.F.	L.F.	R.H.	L.H.			R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.
					1948																
					8. 12	—	—	43*mx*										**1st calving**			
					1949																
23·5	21·8	26·4	28·2	23·9	3. 1	3*m*	5*m*	6*m*	6*m*	—	—	—	—	—	—	—	—				
				21·8	**7. 2**	7*m*	30*m*	5*m*	40*m*	—	—	—	—	—	—	—	—	9·06	9·13	9·22	9·24
23·5	21·5	26·8	28·2	21·4	**17· 2**																
					15. 3	5*m*	4*m*	**60 S**	100*m*	—	—	4	—	—	—	11	—				
25·9	23·7	19·8	30·6	18·3	**16. 3**	—	1*m*	**∞ S**	2*m*	—	—	1	—	—	—	4	—	9·38	9·39	**8·85**	9·48
								←										**R.H. Pen.** (5 × 100,000)			
					25. 3	3*m*	—	10*m*	—	—	—	1	—	—	—	10	—				
26·1	23·9	18·8	31·2	17·9	**30. 3**					—	—	1	—	—	—	8	—	9·11	9·31	**8·22**	9·29
					6. 4	—	3*mx*	3*m*	1*m*	—	—	1	—	—	—	5	—				
					20. 4	1*m*	1*m*	1*m*	3*m*	1	1	2	—	3	2	12	—				
					27. 4	9*m* 6*C*	—	1*m*	1*m*	—	—	1	—	—	—	—	—				
26·8	23·8	17·3	32·1	19·0	**2. 5**	2*m*	—	1*m*	3*mx*	—	—	2	—	—	—	3	—	9·39	9·46	9·21	9·24
					5. 5	1*m*	1*m*	1*m*	9*m*	—	—	1	—	2	—	13	—				
26·5	23·8	16·6	33·1	16·3	**8. 6**	5*m*	—	6*m*	15*mx*	—	—	1	—	3	—	12	—	9·26	9·37	**8·87**	9·33
																		Dry 18. 7. 49			

Table 9. *Clara* 10

An example of a subclinical *Staphylococcus* infection starting in the dry period that reduced the yield and quality of milk of the affected quarter.

Data collected in parts of the fifth and sixth lactations are shown in the table.

Though this cow had a *Streptococcus agalactiae* infection in her R.F. quarter in the third lactation she was free from demonstrable infections, other than *Micrococcus*, in her fourth and fifth lactations. In the following dry period she became infected with staphylococci in the R.F. quarter (see table), and this infection persisted throughout the sixth lactation without causing any noticeable clinical symptoms. The R.H. quarter also became infected with staphylococci in the sixth month of this lactation (not shown in the table), but in this case clinical symptoms developed.

From the table it can be seen that the yields of the individual quarters were normal at the end of the fifth lactation, the L.F. and R.F. quarters giving 30 and 32% respectively of the combined yield of the hind quarters. The subclinical *Staphylococcus* infection which developed in the dry period reduced this proportionate yield of the R.F. to 26% at calving, and there was a progressive fall to 15% when the cow had been in milk for 4 months.

The indirect tests made on the foremilk of the R.F. quarter were constantly positive, and in contrast with the results for Campion 12 the solids-not-fat percentage was significantly lower than would be expected in a normal quarter and probably below 8% for most of June and July. The electrical conductivity determined on the full yield of this quarter (not shown in the table) was also constantly abnormal.

The table also shows interesting observations on the yield and quality of the milk produced by the L.F. quarter. Though no infection could be demonstrated in the L.F. quarter it secreted milk of abnormally low solids-not-fat percentage after the fourth month of the fifth lactation. This was also observed in the first 2 months of the sixth lactation. Throughout this period the indirect tests made on the foremilk were negative, though they became positive in the second month of the sixth lactation. The milk production of the quarter decreased from 34% of the two hind quarters at calving to 28% 4 months later.

This example of an apparently non-infected quarter falling in yield has also been observed with a few other cows, though its occurrence is not common. These quarters very often show no reaction to the indirect tests carried out on the foremilk, while their bulk milks may have a low solids-not-fat percentage and give very abnormal electrical conductivity readings.

It should be noted that the solids-not-fat percentage of all quarters fell below 8·5 in the second half of both the fifth and sixth lactations.

The *Micrococcus* infection in the L.H. quarter did not appear to affect either the relative yield of the quarter or the Whiteside and conductivity results.

Table 9. *The fifth and sixth lactations of Clara* 10 (*Shorthorn*)

Individual quarter yields (lb.) (%)				Daily milk yield (lb.)	Date	Bacteriological results				Whiteside test				Conductivity difference				Solids-not-fat %			
R.F.	L.F.	R.H.	L.H.			R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.	R.F.	L.F.	R.H.	L.H.
20·3	19·1	31·0	29·6		1948																
					17. 6	30*m*	5*m*	70*m*	75*m*	—	—	—	—	—	—	—	—	8·40	8·31	8·38	8·29
					10. 7	4*m*	5*m*	8*m*	50*m*	—	—	—	—	—	—	—	—				
				35·0	**29. 7**	—	—	1*m*	50*m*	—	—	—	1	—	—	—	—	8·87	**8·43**	8·97	8·67
					22. 7	2*m*	3*m*	40*m*	300*m*	—	1	—	—	—	4	—	—				
19·6	18·6	32·5	29·3	35·0	**23. 9**	—	—	50*m*	75*m*	—	—	—	—	—	—	—	—	8·38	**7·90**	8·32	8·34
					1949																
					14. 1	7*mx*	8*mx*	5*m*	100*m*	3	2	4	3					**Dry 14. 1. 49**			
					21. 1	—	1*m*	30*m*	600*m*												
					4. 2	∞ **S**	3*m*	100*mx*	60*m*									**Dry period**			
					28. 3	∞ **S**	5*m*	—	100*m*												
					31. 3	∞ **S**	2*m*	—	150*m*												
15·8	21·4	32·2	30·6		6. 4	**120 S**	—	1*m*	20*m*	4	—	1	4					**6th calving**			
					12. 4	**200 S**	1*m*	1*m*	2*m*	2	—	1	—	2	—	—	—				
				40·5	**20. 4**	**170 S**	1*m*	1*m*	—	2	—	—	—	11	—	—	—	**8·70**	**8·90**	9·31	9·47
13·5	20·2	34·1	32·1	49·6	**9. 5**	**150 S**	—	2*m*	2*m*	1	—	—	—	9	—	—	—	**8·29**	**8·77**	9·07	9·12
12·2	19·8	35·8	32·1	47·8	**8. 6**	**350 S**	1*m*	70*m*	300*m*	1	—	—	—	9	3	—	—	**7·86**	**8·40**	8·90	8·73
					6. 7	**120 S**	1*m*	10*m*	200*m*	1	1	1	1	17	6	3	—	**7·56**	8·26	8·48	8·45
12·3	19·3	35·5	33·0	32·6	**8. 8**	∞ **S**	—	30*mx*	400*m*	1	1	1	1	15	6	—	4	**8·03**	8·48	8·61	8·48

Table 10. *Peach* 25

An example of quarters infected with staphylococci during the dry period, becoming clinical at calving and showing a serious reduction in yield.

Part of the third and fourth lactations are shown in the table.

This Shorthorn cow was free from infection in her second and third lactations, except for the constant presence of micrococci in two quarters. She completed her third lactation with normal quarter yields.

During the dry period new infections occurred in three quarters. The L.F. quarter became infected with micrococci which were replaced by haemolytic staphylococci. The R.H. and L.H. quarters became infected with *Streptococcus uberis*, but this organism did not persist in the R.H. and was replaced by haemolytic staphylococci in the L.H. quarter.

The L.F. and L.H. quarters were abnormally hard and swollen on the day of calving, though the secretion of the L.H. only was slightly discoloured, becoming very abnormal in appearance within a few days. Clots were not apparent in the secretion from the L.F. quarter until the fourth day post-partum. At the height of this clinical attack the infection appeared to be systemic. The cow would not eat her normal food and rapidly lost condition.

Following penicillin treatment of the two affected quarters the staphylococci were eliminated from the L.H. This quarter had practically ceased secreting and remained functionless for the rest of the lactation. The L.F. quarter made a clinical recovery during treatment but was slightly swollen 3 weeks later, and the staphylococci were subsequently eliminated by a repetition of the previous treatment. The yield of this quarter, which was the same as the R.F. quarter at the end of the previous lactation, had fallen to 43% of that of the R.F. quarter 3 weeks post-partum and remained between this figure and 36% for the following 4 months when it became infected with *Str. agalactiae* (not shown in the table).

The Whiteside and electrical conductivity readings of the milk from the L.F. quarter were generally abnormal during the 3 months the quarter was apparently free from infection, but the solids-not-fat percentage was normal and was in fact barely abnormal a month post-partum.

We have no explanation for the high conductivity and low solids-not-fat percentage of the milk from the R.H. quarter in September 1948 and from the R.F. quarter in July 1949. In the former case there was a micrococcal infection which possibly could have been the cause, but in the latter there was no known infection. The Whiteside results were also very abnormal on both occasions. Temporary infections could possibly have been the cause, but this seems unlikely, as such abnormal readings might be expected to be associated with a marked effect on milk yield.

On 26 April the R.H. quarter was horned. It became swollen and blood occurred in the milk on the following day. No infection appeared to derive from this injury.

The results may indicate compensation by the non-infected quarters after the severe damage to the L.F. and L.H. About 2 months after calving the R.F. and R.H. were giving approx. 30 lb. of milk per day, which is the equivalent of a daily yield of 60 lb. per day if all quarters had been normal. Since the maximum daily yields attained in the second and third lactation were 40 and 43 lb. per day, it is unlikely, though not impossible, that she would have given 60 lb. per day in the fourth lactation, and we assume that compensation probably occurred, increasing the yields of the R.F. and R.H. quarters.

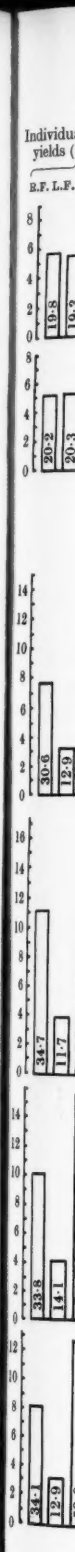


Table 10. *The third and fourth lactations of Peach* 25 (*Shorthorn*)

Individual quarter yields (lb.) (%) R.F.	L.F.	R.H.	L.H.	Daily milk yield (lb.)	Date	Bacteriological results R.F.	L.F.	R.H.	L.H.	Whiteside test R.F.	L.F.	R.H.	L.H.	Conductivity difference R.F.	L.F.	R.H.	L.H.	Solids-not-fat % R.F.	L.F.	R.H.	L.H.
19·8	19·3	30·9	29·9		1948																
					26. 7	300*m*	—	200*m*	—	1	1	1	—	5	5	5	—				
					28. 7	200*m*	4*m*	75*m*	20*m*	1	—	2	—	5	—	11	—				
				28·4	**23. 9**	700*m*	—	250*m*	1*m*	2	1	4	1	6	3	24	—	8·96	9·12	8·85	9·10
20·2	20·3	29·0	30·5	25·0	**19. 10**	150*m*	2*m*	400*m*	5*m*	—	1	—	1	—	8	—	9	9·41	9·30	9·33	9·54
					17. 11	300*m*	—	400*m*	—	—	—	—	—	—	2	—	—	**Dry 2. 1. 49**			
					1949																
					7. 1	∞*m*	∞*m*	∞*m*	6*m* ∞ III	—	—	—	—	—	—	—	—				
					21. 1	∞*m*	∞*m* 17 S	∞ III	∞ S ∞ III	—	—	—	—	—	—	—	—				
					25. 1	∞*m*	150*m* 40 S	∞ III	∞ S	—	—	—	—	—	—	—	—				
					3. 3	∞*m*	∞ S	∞*m*	∞ S	—	—	—	—	—	—	—	—				
30·6	12·9	56·3	0·3		11. 3	80*mx*	∞ S	250*m*	∞ S	—	—	—	—	—	—	—	—	**4th calving**			
					15. 3	∞*m* 5 *S*	∞ S	250*m* 7 *S*	∞ S	—	—	—	—	—	—	—	—				
					←													**L.F., L.H. Pen.** (5 × 100,000)			
				24·5	**4. 4**	4*m* 2 *S*	**60 S**	2*m*	—	—	1	—	1	—	12	—	36	8·76	8·50	8·79	*
					7. 4	2*m*	**200 S**	80*m*	—	—	2	—	1	—	6	—					
34·7	11·7	53·6			22. 4	—	∞ S	350*m*	—	—	1	—	4	—	5	—					
					←													**L.F. Pen.** (5 × 100,000)			
				32·0	**4. 5**	—	2*m*	150*m*	—	—	1	—	—	—	4	—		8·89	8·89	8·92	*
					20. 5	1*m*	1*m*	∞*m*	Blind	—	1	1		—	—	—					
					27. 5	2*m*	—	8 *S* 14*m*	Blind	—	1	—		—	—	3					
33·8	14·1	52·1																			
				30·0	**30. 5**	4*m* 12 *S*	3*m*	130*m*	Blind	1	3	1		—	3	—		8·85	8·73	8·76	*
					9. 6	—	—	150*m*	Blind	—	1	1		—	5	3					
34·1	12·9	53·0		23·9	**19. 7**	—	20*m*	400*m*	Blind	3	3	1		13	6	—		**8·39**	8·58	8·70	*
				17·0	26. 9	—	1*m*	300*m*	250*m*	—	1	3		—	5	4		8·92	8·77	8·90	*
					15. 11	200*m*	250*m*	150*m*	300*m*	2	2	2		—	2	—		**Dry 21. 11. 49**			

* Insufficient milk for analysis.

Table 11. *Dorrit* 12

An example typical of most infected quarters which have been treated and cured showing the absence of any marked increase in yield, relative to the other quarters, until the following lactation.

Data collected during the second and part of the third lactation are shown in the table.

During her first lactation this cow had a clinical *Staphylococcus* infection in her L.H. quarter. The staphylococci persisted through the dry period, and at the start of the second lactation the quarter was clinical, being very swollen, with the secretion discoloured and containing clots.

The infection was cured by penicillin treatment in the first week of lactation, but the indirect tests remained positive for 6–8 weeks. The solids-not-fat percentage, which was of course low while the quarter was clinical, was depressed by only 0·5 two days after treatment was completed, and was normal when tested a month later.

The milk yield of the L.H. quarter was considerably depressed at the beginning of the second lactation, and the yield continued to decline relative to that of the R.H. quarter but not relative to that of the forequarters. There were no infections in the subsequent dry period, and the quarter yields were practically normal at the start of the third lactation, the proportion of the daily yield contributed by the L.H. quarter having increased from 12 to 27 %.

It is important to note that although successful treatment of the infected quarter was not followed by an increased yield of this quarter relative to the other three there was an increase in the total yield. This increase in total yield is probably due to two factors: first the systemic effect of the clinical attack which lowers the yield of all quarters is removed by the successful treatment, and second, compensation in the non-infected quarters may have occurred.

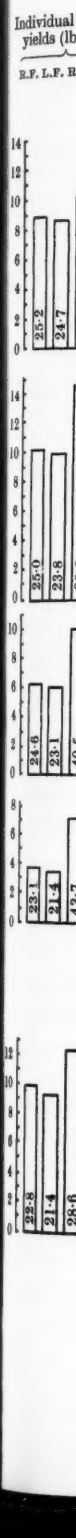


Table 11. *The second and third lactations of Dorrit* 12 (*Shorthorn*)

Individual quarter yields (lb.) (%) R.F.	L.F.	R.H.	L.H.	Daily milk yield (lb.)	Date	Bacteriological results R.F.	L.F.	R.H.	L.H.	Whiteside test R.F.	L.F.	R.H.	L.H.	Conductivity difference R.F.	L.F.	R.H.	L.H.	Solids-not-fat % R.F.	L.F.	R.H.	L.H.
					1948													Dry 25. 7. 48			
					12. 8	5*m*	1*m*	15*m*	**800 S**									} Dry period			
					13. 9	—	—	—	**500 S**												
					25. 9													2nd calving			
					26. 9	10*m*	—	4*m*	**300 S**	—	—	—	3	—	—	4	6				
					29. 9	3*m*	2*m*	5*m*	**1000 S**	2	2	2	4	—	—	—	23				
					30. 9	3*m*	—	3*m*	**600 S**	—	—	—	4	—	—	—	15				
																		← L.H. Pen. (5 × 100,000)			
25·2	24·7	37·5	12·7	35·3	**6. 10**	3*m*	5*m*	—	1*m*	—	—	—	2	—	—	—	5	9·45	9·60	9·51	**9·10**
					9. 10	1*m*	—	—	1*m*	—	—	—	2	—	—	—	7				
25·0	23·8	38·0	13·2		11. 10	6*m*	1*m*	—	1*m*	—	—	—	2	—	—	—	6				
				39·4	**1. 11**	—	3*m*	—	5*m*	—	—	—	1	—	—	—	2	9·21	9·26	9·28	9·25
24·6	23·1	40·5	11·7	34·4	29. 11	3*m*	—	—	1*m*	—	—	—	1	—	—	—	—				
					1949																
				24·4	**27. 1**	4*mx*	5*mx*	—	1*C*	—	—	—	—	—	—	—	—	9·02	8·97	9·19	9·10
23·1	21·4	43·7	11·8	16·1	**30. 3**	20*m*	2*m*	—	6*m*	2	—	—	2	8	—	—	4	9·02	8·81	9·01	**8·72**
					5. 5	—	—	—	—	1	—	—	2								
					28. 6	1*m*	1*m*	60*m*	—	2	2	4	2					Dry 3. 7. 49			
					4. 7	1*m*	1*m*	—	—									} Dry period			
					2. 9	2*m*	—	—	12*m*												
					9. 9	6*mx*	—	4*mx*	—	—	—	—	—					3rd calving			
					16. 9	2*m*	—	—	—	1	1	2	2								
22·8	21·4	28·6	27·2	43·5	**19. 9**	—	2*m*	—	1*m*	1	1	2	2	—	—	—	—	9·22	9·14	9·33	9·18
					19. 10	—	—	4*mx*	3*m*	—	—	—	—	2	—	—	—				

KEY TO TABLES

R.F., L.F., R.H., L.H.: right front, left front, right hind and left hind quarters.

Individual quarter yields. The mean daily yield of each quarter is shown in the block diagrams and the proportionate yields of quarters as percentages in each block figure. These percentages are calculated from the sum of the four consecutive quarter milkings, commencing on the date shown in heavy type. A cow was said to have normal quarter yields if the proportionate daily yields of the two front quarters and also of the two hind quarters were approximately the same.

Bacteriological results

All colonies on the blood plates were recorded, and the numbers given in the tables refer to the total number of colonies on the plates other than the common udder diphtheroids. The letter following each number signifies the type of micro-organism as follows:

I, *Streptococcus agalactiae.*
II, *Str. dysgalactiae.*
III, *Str. uberis.*
S, Staphylococci (coagulase positive).
} Infections caused by these organisms are given in heavy type.

m, Micrococci (or coagulase negative 'Staphylococci').
C, Coliform bacteria.
mx, Very mixed flora. Generally caused by careless sampling.
∞, Innumerable.

Clinical symptoms

A quarter was said to be clinical if the milk showed clots and/or there was marked inflammation and swelling of the quarter. This condition is indicated by a thick line in the appropriate bacteriological results column, and the severity of the attack is described in the accompanying notes.

Whiteside tests

The results of the Whiteside test were graded as 1, 2, 3 or 4. Grade 1 indicates a slight but definite positive result to the test, while grade 4 was usually found when the quarter was clinical or approaching this state.

Conductivity

The electrical conductivity of the milk from each quarter of a cow was compared. The lowest reading was taken as the reference, and the difference, if 2 or more, between this conductivity reading and that for the other quarters is shown in the 'conductivity difference' column. Differences greater than 2 ($\times 10^{-4}$ mho) have been taken to the nearest whole number.

Solids-not-fat percentage

The solids-not-fat percentages for each quarter are given and are in heavy type when considered to be abnormal.

Treatment

The penicillin treatment is recorded in the right-hand column. Thus R.F.Pen. ($5 \times 100{,}000$) means that the right front quarter was infused with 100,000 units of calcium penicillin* after five consecutive afternoon milkings.

* Supplied in collapsible tubes by Burroughs Wellcome and Co., London.

DISCUSSION

It is now accepted that the total loss in milk yield caused by mastitis cannot be measured directly by estimating the relative loss in production of the infected quarters. Compensation by the uninfected quarters is known to occur (18, 19), though this phenomenon has not been fully investigated. The studies presented in this paper are intended to indicate not the total loss in milk but to illustrate the damage to milk-secreting tissue which can occur following intramammary infections of varying degrees of severity and caused by different micro-organisms. They also show the effect of treatment following a clinical attack in different periods of a lactation, and the amount of regeneration that may occur in the remainder of lactation and during the succeeding dry period. The case histories of the eleven cows were selected to show a wide range of effects, and no attempt has been made at this stage to show the proportion reacting in any particular way.

The results in Tables 1–5 indicate that the infections generally described as subclinical and caused by streptococci or staphylococci may or may not result in progressive damage to udder tissue. Whilst Campion 17 (Table 1), Campion 15 (Table 2) and Pandora (Table 6) show a marked progressive drop in milk yield and quality, Medlar (Table 3), Flora 63 (Table 4) and Campion 12 (Table 5) show no effect, even though the infections were with similar organisms and persisted over periods of many months. Though the reduction in yields of the former cows was not marked in a short period the damage was quite severe over a period of several months. That the infections in the cases of the three latter cows were true infections is shown by the presence of the organism at each test of the affected quarter, its absence from one or more other quarters of the same cow and the frequent indications of abnormality revealed by the Whiteside and conductivity tests on the milk of the infected quarter.

Presumably the infections which did not reduce milk yield were either restricted to connective tissue, localized in one or more foci in secretory tissue, or confined to small accessory glands reported as being present in the walls of some teat sinuses (20).

Infections which have no apparent effect on the relative yield of a quarter do not generally reduce the solids-not-fat percentage nor increase the electrical conductivity of the total production of the quarter, though the first ounce of foremilk is usually positive to the Whiteside and conductivity tests and sometimes strongly positive.

In studies of this type it is necessary, to avoid a false interpretation of the results, to have complete knowledge of the previous mastitis history of the cows. For instance, if the cow Pandora (Table 6) had not been tested until March or April 1949, the fall in yield of the left hind quarter might have been interpreted as the effect of the subclinical infection present at that time. The table shows, however, that most of this reduction was caused by the clinical attack occurring at the end of January 1949. Furthermore, we have records (not shown here) that some clinical attacks, so mild or of such short duration as to escape notice, may cause very severe reductions in yield.

Tables 6–8 reveal the effects of clinical mastitis of short duration, i.e. a mastitis in which the clinical state has been terminated by treatment with penicillin. Usually there was an immediate fall in milk yield, the results of the Whiteside and conductivity tests became more positive and there was a significant fall in the solids-not-fat percentage. Though this is the most common picture there are exceptions, e.g. the second clinical attack of Pandora (Table 6) and that of Flora 63 (Table 4) produced no measurable fall in

yield, and the serious decrease in yield caused by the very mild clinical attacks mentioned above, show the variations in effect which occasionally occur. Successful penicillin treatment was not normally followed by a recovery in milk yield of the affected quarter, either immediately or in the rest of that lactation. The solids-not-fat percentage and the Whiteside and conductivity tests sometimes revert to normal soon after treatment, though they usually remain abnormal if the cow is approaching the end of the lactation or if the infection has reduced the yield of the quarter to a very low level. The results given in Table 11 confirm, in a very striking manner, the earlier findings of Edwards & Brownlee (7). Here the severely damaged secretory tissue of the left hind quarter of Dorrit 12 showed complete regeneration during the dry period. We have frequently observed this phenomenon when the damaged quarter has been free from infection during the dry period.

We have reported in a previous communication (21) that there is a very high new infection rate in the early dry period. Tables 10 and 11 give illustrations of cows infected in this way and show the very marked effect of these infections on the milk yield in the following lactation. Though the clinical attacks of Peach 25 (Table 10) produced the most marked effects, the subclinical dry-period infection of Clara 10 (Table 9) also produced a serious reduction in yield. These two results are indicative of the serious course which dry-period infections may take.

None of the tables given in this paper provides conclusive evidence of compensation. There is a possibility, however, that compensation occurred in at least two cases, and these have been commented on. It is hoped that a full analysis of all our data will give further evidence on the compensation that does occur, though this may need separate investigation.

From the tables presented above it can clearly be seen that, provided that great care is taken in the collection of milk samples, the methods of diagnosis used provide data which are remarkably consistent. Apart from one case (Niobe R.H. quarter) the infections were readily diagnosed and the bacteria repeatedly isolated at almost every test. Similarly, the results show the great value of the indirect tests in providing confirmation of the bacteriological findings in spite of the large number of positive results which these tests give in late lactation or with 'light' quarters.

In many cases when the yield of a previously non-infected quarter fell below 5–6 lb. a day the foremilk gave abnormal reactions to the indirect tests though no bacteria were found, e.g. the R.F. quarter of Campion 15, Pandora, Pippin 12 and Peach 25, the L.F. quarter of Campion 17 and the R.H. quarter of Campion 12. That most of these abnormalities occurred in forequarters is understandable, as the yields of the forequarters naturally fall to a minimum sooner than those of the hind quarters. It is probable that such changes have no connexion with infection, though they may be related to the method of machine milking. Van Rensburg (22), however, found that the forequarters were more 'abnormal' in composition than the hind quarters, and it is possible that most of his figures for abnormality occurred when the yields of the forequarters were below 5 or 6 lb. a day. We also have some evidence that forequarters 'dry off' more quickly near the end of lactation than the hind quarters.

There was no evidence that physical and chemical changes in the composition of the milk preceded bacterial invasion.

From the results reported in this paper and others not yet reported it may be that some of the abnormalities referred to in the comprehensive studies of Hastings & Beach (1, 2) and

Van Rensburg (22), have been caused by *Staphylococcus* and *Micrococcus* infections or have resulted from dry-period infections. These workers reported the results of tests on quarters which gave consistently high chloride and catalase results, and in some cases showed marked reductions in yield in the absence of specific bacteria. While we have encountered a number of quarters which showed a fall in yield, relative to the other quarters, in the absence of any demonstrable bacteria, such quarters have been relatively few and could in some cases be attributed to transitory clinical infections. One exception, however, was the L.F. quarter of Clara 10 (Table 9), in which the abnormality consisted of low solids-not-fat in the 'full-yield' samples while the foremilk gave little indication of abnormality and no infection was found. In many of the cases reported by Hastings & Beach and by Van Rensburg however, bacteria were present, though they were regarded as part of the normal non-specific flora, and in a few instances clinical symptoms were observed.

It is evident from our studies, some of which have been reported in this paper, that one type of organism (e.g. micrococci and staphylococci) may infect more than one quarter of the same udder, causing little or no irritation in one gland and marked abnormalities in another; also, that a subclinical infection in one quarter will not prevent a new infection of the same type causing marked clinical symptoms in another quarter.

SUMMARY

A study has been made of the effect of udder infections on the proportionate yield and composition of the milk of individual quarters of over 100 cows, and the data from eleven of these cows have been presented.

Typical examples have been given showing that subclinical *Streptococcus* and haemolytic *Staphylococcus* infections may persist for months and either cause a gradual reduction in the proportionate yield and solids-not-fat percentage of a quarter or have no measurable effect, and that both types of reaction may occur simultaneously in the same udder.

Examples also show that following penicillin treatment of clinical cases caused by streptococci and staphylococci there is little recovery in the proportionate yield of the treated quarter until the following lactation, when recovery may be complete.

We wish to acknowledge the help of Dr S. J. Rowland who arranged for the chemical analysis of the milk samples and Dr A. T. R. Mattick and Mr A. S. Foot for their advice and criticism.

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417. KEEPING QUALITY AND RAW-MILK GRADING

FOREWORD BY

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I. DYE TESTS AS MEASURES OF THE KEEPING QUALITY OF MILK

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(With 9 Figures)

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FOREWORD

The experiments analysed in this series of papers were undertaken as the result of a discussion on methods of measuring the keeping quality of milk, at a Conference of Advisory Bacteriologists of the Ministry of Agriculture and Fisheries.

Owing to the disturbance and difficulty following the outbreak of war and the dislocation of handling and distribution, a serious decline in the keeping quality of raw-milk supplies was threatened.

After a field trial in several counties a National Milk Testing and Advisory Scheme was established with the object of revealing to producers, faults in milk production leading to loss of keeping quality. The resazurin test was used to measure keeping quality, in one form as a rejection test and in the other as a routine test which placed milk in several categories. The rejection test soon proved valuable on the milk platform as an objective method of segregating milk with poor potential or actual keeping quality, but it was felt that before the routine resazurin test passed from the tentative to the official stage as a means of grading, it was necessary to obtain further information on its relationship to the actual keeping quality of milk.

For many years the methylene-blue reduction test, which replaced the plate count, had been used for the official grading of milk, and it was felt that the relationship of this test to keeping quality should be examined at the same time.

When results of estimating keeping quality by the alcohol precipitation test, the clot-on-boiling test and a tasting test were available it was deemed desirable to determine the suitability of these tests for the grading of milk and possibly for differential payment.

With these objects in view a Committee to report to the Conference of Advisory Bacteriologists was appointed as follows:

Dr A. T. R. Mattick (Chairman).

Dr J. G. Davis, Capt. H. Barkworth, Mr S. B. Thomas with Mr A. Rowlands and Dr F. Yates as co-opted members (Dr J. G. Davis resigned shortly after his appointment, on leaving the public service).

Dr F. Yates undertook to supervise the design of the experiments and their statistical analysis.

The experiments themselves were carried out in three widely separated areas in England and Wales, at the Midland Agricultural College, under Mr A. Rowlands, who, however, was followed early in the work by Mr C. A. Scarlett; at University College of Wales, Aberystwyth, under Mr S. B. Thomas and at the National Institute for Research in Dairying, first under Dr J. G. Davis and then under Mr A. Rowlands.

In consultation with Dr F. Yates and his colleagues, Capt. H. Barkworth and Miss Z. Hosking were responsible for much computation of data and their statistical presentations.

In many similar investigations the value of the data has been seriously impaired because no observations were made during the night to record the actual end-points of the various tests. It was felt that such end-observations must be made in these experiments. This decision involved the participants in day and night observations for long periods, and it is proper to pay tribute to their work. Without their loyal co-operation it would have been impossible to follow the experimental procedures which were laid down.

The following workers took part:
Midland Agricultural College:
Miss Anne M. Blomfield, B.Sc.
University College of Wales:
Miss A. T. Stewart, B.Sc.
Miss Anne M. Hughes, B.Sc.
Miss D. Ellison.
National Institute for Research in Dairying:
Mr F. Wilby, B.Sc.
Mr N. Gruber.
Miss Valerie Border.

The Ministry of Agriculture and Fisheries provided grants to defray the cost of the experiments.

INTRODUCTION

The measurement of the keeping quality of milk has been the subject of many publications, but in fact the ordinary consumer, being assured of its safety, demands little more than that milk shall remain palatable and uncurdled until consumed. Keeping quality may, therefore, be defined as the length of time from production or other defined period during which milk remains in a usable condition. One method of assessment is to store samples at some constant temperature and to taste them at regular intervals until they become unpalatable, relating this period to reasonable temperature conditions during household storage.

But the sense of taste is very subjective. It is impossible to define the end-point of acceptability and to ensure that its assessment by different individuals does not vary; personal bias in the judgment of the end-point is unavoidable and leads to large errors. Results obtained by Mattick, Hiscox & McClemont(1) emphasize the unreliability of determining keeping quality by taste. Observations on samples of raw milk kept at 15·5° C. and tasted at 2 hr. intervals, without knowledge of the results recorded at previous observations, showed that it was not uncommon for the same individual to record a sample as tainted at one observation and to reverse his decision at a subsequent observation; the time of first occurrence of off-flavour in the same sample was seldom the same with the three observers and usually differed by one or more 2 hr. intervals. Using results from three different laboratories a similar conclusion as to the unreliability of tasting was reached by Barkworth, Irwin & Mattick(2). In the present series of investigations, in which samples stored at $18 \pm 1°$ C. were tasted at 4 hr. intervals, there were also numerous instances where samples were recorded as tainted at one observation and as doubtful or sweet at a subsequent observation 4 hr. later.

One of the objects of the present series of investigations was to assess the suitability of dye tests, in particular the resazurin test, as measures of keeping quality. For this purpose it was essential to use, as a measure of the end-point of keeping quality, an objective method with defined limits of error; taste is clearly unsuitable.

Two objective methods, clot-on-boiling (C.O.B.) and precipitation with 68% alcohol (A.P.T.) were selected for further study. Data on the reproducibility of the results using these two tests are given in Appendix 1 (p. 183), and results of more exhaustive investigations of the alcohol test in Appendix 2 (p. 186). Highly reproducible results were obtained with both tests, and they were therefore used as objective measures of keeping quality in the extensive series of experiments which will now be considered.

EXPERIMENTAL

The experiments were carried out at three centres, *K* in the south of England, *L* in Wales, and *M* in the Midlands, and covered both 'winter' and 'summer' seasons. The winter season extended from October 1944 to April 1945, and the summer season from May to September 1945. Approximately equal numbers of samples of evening and morning milk were tested at each centre in each season. The numbers are given in Table 1.

Table 1. *Number of samples tested at each centre*

	Winter season*		Summer season†	
Centre	Evening	Morning	Evening	Morning
K	44	30	21	35
L	31	32	38	35
M	28	24	32	36
Total	103	86	91	106

* October 1944 to April 1945 inclusive. † May to September 1945 inclusive.

SOURCE AND STORAGE OF SAMPLES

In experiments of this kind it is essential that the samples examined shall represent milk of the widest possible range of bacteriological quality. To this end, therefore, milks from farms of which the type of production was known from previous records were selected for study.

One-quart samples from individual producers were taken either at farms or on delivery to creameries. The evening samples were taken on the morning after production, and the morning samples on the morning of production. All samples were received at the testing laboratories before 10 a.m.

On arrival at the laboratory each quart sample was divided into two 1 pint (*c.* 560 ml.) subsamples in screw-topped blood transfusion bottles for storage in a shade atmospheric box (3) until testing began. Testing of one of the evening subsamples (section A) began at 10 a.m. and of the other subsample (section B) at 4 p.m. on the day after production. The testing of one of the morning subsamples began at 4 p.m. (section C) on the day of production, and of the other at 10 a.m. (section D) on the day following production. Thus the periods of storage from the time of milking (taken as 3 p.m. for evening milk and 6 a.m. for morning milk) before tests were begun were 19, 25, 10 and 28 hr. respectively for the subsamples in the four sections A, B, C and D. The A and C sections correspond to the prescribed times and conditions for testing designated raw milk in England and Wales (4) and the B and D sections to the times for testing by the routine resazurin test (3).

Plan of experiments

	Evening milk		Morning milk	
Approx. time of production ...	3 p.m.		6 a.m.	
Subsamples	Section A	Section B	Section C	Section D
Period of pre-test storage (hr.)	19	25	10	28
Tests begun at	10 a.m.	4 p.m.	4 p.m.	10 a.m.

Subsamples in each section examined by the following tests:

Keeping quality at 18° C. Observations made at 4 hr. intervals using taste, 68% brom-cresol purple/alcohol (A.P.T.), and clot-on-boiling (C.O.B.).

Titratable acidity at 18° C. Tests at 4 hr. intervals until end-point of C.O.B. test reached.

Methylene blue test. (*a*) at 18° C. with observation and inversion of tubes at 2 hr. intervals; (*b*) at 37·5° C. with observation and inversion of tubes at ½ hr. intervals.

Resazurin test. (*a*) at 18° C. with observation and inversion of tubes at 2 hr. intervals; Lovibond disk number recorded at each observation; (*b*) at 37·5° C. with observations as for the test at 18° C. but at ½ hr. intervals.

Colony count. Triplicate plates; one set incubated at 22° C. for 5 days, one at 30° C. for 4 days, and one at 37·5° C. for 3 days.

After storage for the prescribed times of the subsamples representing the four sections A, B, C and D, 10 ml. quantities were withdrawn with sterile pipettes and transferred to $6 \times \frac{5}{8}$ in. sterile test-tubes for the methylene-blue and resazurin tests at 37·5 and 18° C.; colony-count determinations, with incubation of plates at 22, 30 and 37·5° C., were also made. The blood-transfusion bottles containing the remainder of each subsample were then placed in a thermostatically controlled water-bath maintained at 18 ± 1° C. for the keeping quality tests.

METHODS OF MEASURING THE END-POINT OF KEEPING QUALITY

At the time of transfer to the water-bath, and thereafter at 4 hr. intervals, a portion of each subsample was withdrawn for testing by taste, C.O.B. and 68% alcohol. Acidity tests were made at the time of transfer to the water-bath and at every observation time after the brom-cresol-purple (B.C.P.) indicator in the alcohol test showed a change in colour. The keeping quality by the first three tests was taken as the time in hours after transfer to the 18° C. bath until the end-point was reached.

Keeping quality by taste. The methods of the Ministry of Agriculture (5) were used for tasting samples and recording the results. In this method, the presence of a distinct taint, *T*, which is not sufficient to render the milk unfit for use, is taken as the end-point; when a reading of doubtful, ., is followed by condemned, *X*—a distinct taint rendering the milk unfit for use—the former is taken as the end-point; when a reading of sweet, −, is followed by condemned, *X*, the end-point is taken as having occurred midway between the two observations.

All samples were tasted at 4 hr. intervals until condemned, *X*, or until the end-point with the C.O.B. test.

Since, by definition, a record of *T* is made whenever a sample is deemed to possess an off-flavour, it frequently happens that *T* is recorded at several successive observations; not uncommonly a record of *T* was followed by . or even − in our experiments. The first *T* was taken as the end-point irrespective of the results obtained at subsequent observations.

Clot-on-boiling (C.O.B.). Approximately 2 ml. of milk in a $6 \times \frac{5}{8}$ in. test-tube was placed in boiling water for 5 min. The tube was then removed from the bath, and, without shaking, was tilted to expose a thin film of milk. Samples showing definite evidence of curdling, or the presence of a discrete precipitate not restricted to the cream layer were recorded as +; clotting or a heavy precipitate were recorded as ++; a doubtful result was recorded .. The first recorded + if preceded by . or − was taken as the end-point; when a − reading was followed by ++ the end-point was taken as having occurred midway between the two readings; when a ++ followed a reading of . the end-point was deemed to have occurred at the time the . was recorded.

Precipitation with neutral 68% *alcohol* (A.P.T.). In this test 68% (v/v) ethyl alcohol containing approximately 0·005% B.C.P. as indicator was used. The alcohol solution was

prepared as follows: Dissolve 0·05 g. B.C.P. in 500 ml. 95% alcohol; add N/9-NaOH until the colour of the indicator changes through grey-green to grey-purple; add distilled water until the specific gravity at 15·5° C. is 0·895.

For testing, 1 ml. of the alcohol/B.C.P. mixture was added to 1 ml. of milk in a $6 \times \frac{5}{8}$ in. test-tube. A slight precipitate adhering to the wall of the tube was taken as the end-point. For determining the end-point the same rules were followed as with the C.O.B. test.

Titratable acidity. To 10 ml. of milk add 1 ml. of 0·5% phenolphthalein (alcoholic solution) and titrate to a faint pink end-point with N/9-NaOH; record the results as percentage lactic acid.

METHODS OF APPLYING THE DYE TESTS

For the methylene-blue and resazurin tests 1 ml. of the standard dye solutions was added to 10 ml. of milk in a sterile $6 \times \frac{5}{8}$ in. test-tube and the tube stoppered. The tubes were placed in thermostatically controlled water-baths maintained at $37{\cdot}5 \pm 0{\cdot}5$ or $18 \pm 1°$ C. In the tests at 37·5° C. the tubes were observed and inverted every $\frac{1}{2}$ hr., and at 18° C. every 2 hr. Complete reduction of methylene blue (4) was taken as the end-point. For resazurin the Lovibond disk number was recorded at each observation. A special disk ranging from 6 to 1 in $\frac{1}{2}$ disk intervals was used; colours not matching the disk colours exactly were estimated to the nearest $\frac{1}{4}$ disk. A disk reading of 0 was taken as the end-point.

COLONY-COUNT DETERMINATIONS

Plates were prepared in triplicate using yeastrel milk agar (4); one set was counted after incubation for 5 days at 22° C., the second after 4 days at 30° C. and the third after 3 days at 37·5° C.

RESULTS

METHODS OF ANALYSIS OF THE RESULTS

The first stage in the statistical analysis of the results was the preparation of scatter diagrams illustrating the relationship between pairs of observations on the same samples. Consideration of these diagrams made clear the main conclusions to be drawn from the data; where necessary standard methods of regression analysis were used to express these conclusions in numerical form. Examination of the data showed that the results for the three centres *K*, *L*, *M* could properly be pooled. Winter and summer results, and the results for the four sections A, B, C, D, were, however, treated separately.

The diagrams showed that the relationship between the different tests was essentially similar in all four sections. Accordingly most of the diagrams which are reproduced are for the morning subsamples in section C, tested after storage at shade atmospheric temperature for 10 hr. before the tests were begun.

DIRECT MEASURES OF KEEPING QUALITY

Relationship between keeping quality results using the subjective tasting test and the objective C.O.B. *and* A.P.T. *tests*

Fig. 1 depicting the relationship between time to C.O.B. and time to *T* at 18° C. for section C winter and summer samples is representative of similar diagrams prepared for the other three sections and for the relationship between time to A.P.T. and time to *T* for the four sections. This diagram shows that the regression between the subjective and the

objective tests is linear, i.e. the increase in the time to reach T associated with unit increase in time to C.O.B. or A.P.T. will, on the average, be the same at all levels of keeping quality. The individual results are, however, scattered rather widely about the average line. This scatter is partly due to the interval between observations, since the true end-point may occur at any moment in the interval between the last negative and the observed end-point. Non-bacterial taints may also influence the scatter of the results, since when they are present taste will measure a property different from that measured by tests which reflect the activities of bacteria, and T may be recorded much earlier than C.O.B. or A.P.T. A scatter of this nature is inevitable in any comparison of subjective and objective tests, and its magnitude will, in general, be determined mainly by the error of the subjective measure used.

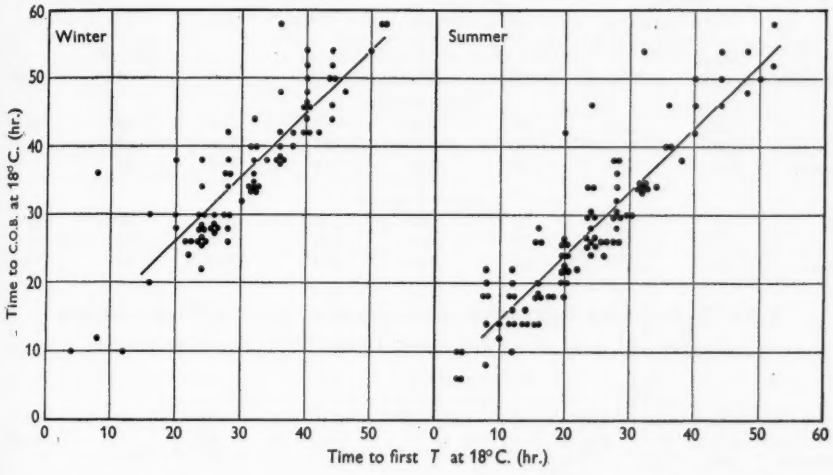

Fig. 1. Relationship between time to clot-on-boiling (C.O.B.) and time to first taint (T) at 18° C. for section C subsamples.

It would be absurd to evaluate the suitability of C.O.B., A.P.T., or any other objective measure of the keeping quality of milk by comparing the results with those obtained by tasting the samples. If any of the objective methods were to be used as a basis of evaluation, any judgment of the accuracy of the tasting method would be of value only to assess the results obtained by a single observer or a group of observers; it need not necessarily be even remotely applicable to another group of tasters.

It is of some interest, however, to examine the general relationship for the samples in the present series of experiments in terms of the interval between time to end-point by taste and by C.O.B. or A.P.T. From the mean values (hr.) for the three tests (Table 2) it may be seen that the end-points occurred in the order, Taint, A.P.T. and C.O.B. Taint occurred 1–3 hr. earlier than A.P.T. in winter, but in summer the two end-points coincided; in winter taint occurred 6–8 hr. before C.O.B. and about 3 hr. before in summer.

Anderson & Wilson (6), who used taste and 68% alcohol (A.P.T.) to measure the end-point of keeping quality (at 16–18° C.) of 2588 raw-milk samples, found that the two end-points coincided with 60·3%, the A.P.T. end-point occurred before taint with 25·5%, and taint before A.P.T. with 14·2% of the samples. They concluded: 'Even if taste was omitted

altogether, because expert milk tasters were not available, the K.Q. of the milk could be gauged fairly accurately by the alcohol test alone in all but 14% of the samples examined.' In Table 3 our own results are presented in the form of a frequency distribution of the difference between times to end-point with *T* and A.P.T. The end-points coincided in a smaller proportion of samples than was found by Anderson & Wilson; these authors made observations at 9 a.m., 3 p.m. and 9 p.m. only, and the longer interval between observations would increase the chance of the end-points coinciding. In a high proportion of our samples the end-points either coincide or occur within two successive observations (±4 hr.).

Table 2. *Mean values (hr.) of times to end-point at 18° C. using first taint (T), 68% alcohol* (A.P.T.) *and clot-on-boiling* (C.O.B.)

	Section A (19 hr. storage)		Section B (25 hr. storage)		Section C (10 hr. storage)		Section D (28 hr. storage)	
End-point	No. of samples	Mean	No. of samples	Mean	No. of samples	Mean	No of samples	Mean
				Winter season				
T	100	28·1	97	26·6	83	31·0	83	26·0
A.P.T.	103	31·5	101	30·1	85	32·6	85	27·4
C.O.B.	103	36·3	103	34·4	84	36·4	84	31·9
				Summer season				
T	82	20·9	77	17·4	100	23·8	80	13·7
A.P.T.	91	20·5	80	17·7	106	24·2	91	13·9
C.O.B.	91	23·8	83	20·4	106	27·6	98	16·4

Table 3. *Frequency distribution of samples according to difference between time to end-point using first taint (T) and 68% alcohol* (A.P.T.)

	T − A.P.T. (hr.)*										
Section	−18 and over	−14 to −16	−10 to −12	−6 to −8	−2 to −4	0	2 to 4	6 to 8	10 to 12	Total	% in range −4 to +4
					Winter season						
A	6	4	8	10	26	27	16	1	2	100	69·0
B	5	5	12	13	19	20	19	3	1	97	59·8
C	1	1	2	12	19	28	18	2	0	83	78·3
D	0	0	6	15	16	25	17	4	0	83	69·9
					Summer season						
A	0	1	1	8	19	28	24	1	0	82	86·6
B	0	2	1	6	17	18	28	2	1	75	84·0
C	0	2	2	13	17	33	32	1	0	100	82·0
D	1	0	5	8	15	24	24	1	0	78	80·8

* Negative values, *T* occurs before A.P.T.; positive values, *T* occurs after A.P.T.

At all seasons, and especially in winter, some samples were deemed to be tainted many hours before they reached the A.P.T. end-point. We have no means of determining to what extent these large differences between the two end-points were artifacts arising from the subjectiveness of tasting, or due to off-flavours of non-bacterial origin with consequently no effect on the A.P.T. end-point. It is perhaps of some significance that ten of the thirteen samples whose A.P.T. end-point occurred 18 or more hours after the first recorded *T* were tested at centre *K*. With five of these ten samples there was considerable uncertainty about the true end-point by taste; there were records of doubtful, ., and with some even of sweet, −, after the first *T*, which was actually deemed to be the end-point. For one of the samples in particular, whose end-point was taken as 12 hr. for *T* and 50 hr. for A.P.T., the

observer on duty during the night recorded *T* at 12, 16 and 20 hr., − at 36 hr. and . at 40 and 44 hr., whereas the observer on duty during the day recorded − at 0, 4, 8, 28 and 32 hr., and . at 24 hr.; the sample was finally condemned, *X*, at 48 hr. and reached the A.P.T. end-point, + +, at 52 hr. We have reason to believe that with the majority of these thirteen samples the off-flavours were non-bacterial in origin, for, despite their low colony counts and long reduction times initially, *T* was recorded so early in the life of the samples that the off-flavours were unlikely to be the result of bacterial metabolism.

While it is clear, for the reasons already given, that taste is unsatisfactory for measuring the end-point of keeping quality, objective methods such as A.P.T. or C.O.B. cannot entirely replace taste in the day-to-day commercial contacts of producer, distributor and consumer.

Relationship between times to end-point using A.P.T. *and* C.O.B. *as measures of keeping quality*

For the subsamples in each of the four sections A, B, C and D, results of keeping quality measured under identical conditions using A.P.T. and C.O.B. as the end-points were available. The relationship was essentially similar for subsamples in each of the four sections and is exemplified in Fig. 2, where the time to A.P.T. is plotted against the time to C.O.B. for section C of the winter and summer samples.

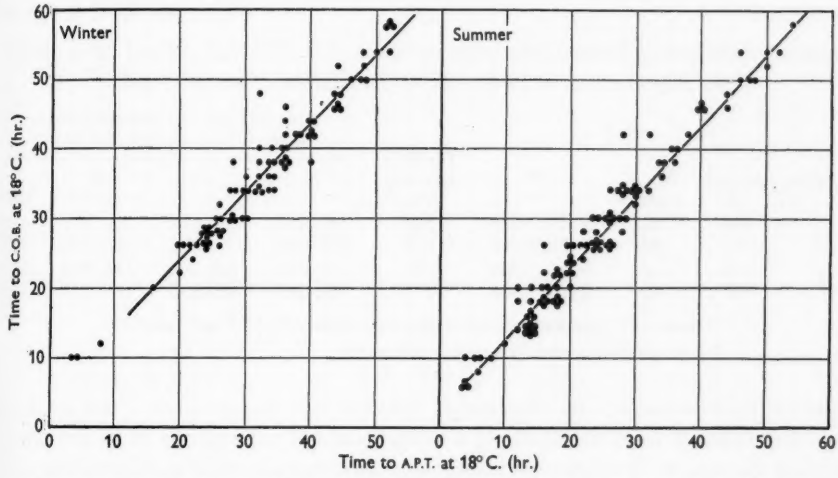

Fig. 2. Relationship between time to C.O.B. and time to precipitation with 68 % alcohol (A.P.T.) at 18° C. for section C subsamples.

The results have been analysed by obtaining the regression of time to A.P.T., y, on time to C.O.B., x, in the form $y = y_0 + b(x-30)$.* The value of 30 hr. corresponds approximately to

* Regression lines are usually obtained in the form $y = \bar{y} + b(x-\bar{x})$, $\bar{x}$ and $\bar{y}$ being the means of the x and y values respectively; and b, the regression coefficient, denotes the increase in y corresponding to unit increase in x. The variance of a value of y on the line corresponding to any x is as follows: $V(\bar{y}) + V(b)\,(x-\bar{x})^2$, where Vb is the variance of the regression coefficient. It follows that if the two values of y on two regression lines, but corresponding to the same value of x, are to be compared, the $Vb(x-\bar{x})^2$ terms cause the two y values to differ in precision. None of the $\bar{x}$ values differed greatly from 30 hr. C.O.B. In each case, therefore, the term $(30-\bar{x})^2 Vb$ was small, and comparisons of the y's corresponding to 30 hr. C.O.B. were satisfactory. With the regression lines in the form $y = y_0 + b(x-30)$ the required comparison became simply a comparison of the y_0 values, for when $x=30$, $y=y_0$.

the observed mean C.O.B. time of the samples in the present series of experiments, and y_0 in the equation is the value for A.P.T. which corresponds to a value of 30 hr. for C.O.B. Table 4 contains the values of y_0 and b; the table also contains the residual standard error, s, of A.P.T. which is a convenient measure of the spread of the relationship.

Table 4. *Relationship between time to precipitation with* 68% *alcohol* (A.P.T.) *and time to clot-on-boiling* (C.O.B.) *for samples at* 18° *C.*

Section	No. of samples	b*	Residual standard error, s	Time to A.P.T., y_0, corresponding to time to C.O.B. of 30 hr. for centre: *K*	*L*	*M*	Mean
A: Winter	103	0·947±0·027	3·21	(44) 26·6	(31) 24·4	(28) 25·3	25·4
Summer	91	0·975±0·022	2·13	(21) 26·1	(38) 27·3	(32) 25·8	26·4
B: Winter	101	0·957±0·033	3·87	(44) 26·1	(31) 24·1	(26) 25·2	25·1
Summer	80	0·957±0·037	2·89	(19) 26·1	(30) 27·1	(31) 25·7	26·3
C: Winter	84	0·953±0·028	2·56	(30) 26·7	(30) 26·6	(24) 26·2	26·5
Summer	106	0·938±0·020	2·28	(35) 26·5	(35) 27·2	(36) 25·9	26·5
D: Winter	84	0·926±0·024	2·25	(29) 26·3	(31) 24·8	(24) 27·2	26·1
Summer	91	0·944±0·027	2·65	(30) 25·9	(28) 26·2	(33) 25·5	25·8

Figures in ( ) denote number of samples contributed by each centre.
* b = regression coefficient for combined centres.

Table 5. *Relationship between time to precipitation with* 68% (A.P.T.) *and* 50% *alcohol and time to* C.O.B. *at* 18 *and* 22° C. *for samples of morning milk*

Incubation temp. (° C.)	Alcohol (%)	No. of samples	b*	Residual standard error, s	Time to A.P.T., y_0, corresponding to time to C.O.B. of 30 hr. for centre: *K*	*L*	*M*	Mean
18	68	64	0·948±0·026	2·04	(21) 27·5	(20) 28·3	(23) 25·1	27·0
	50	66	0·973±0·019	1·53	(21) 30·0	(20) 30·1	(25) 28·2	29·5
22	68	59	0·961±0·027	1·35	(19) 28·0	(18) 28·3	(22) 26·2	27·5
	50	60	0·965±0·023	1·15	(19) 29·9	(18) 30·2	(23) 28·7	29·6

Figures in ( ) denote the number of samples contributed by each centre.
* b = regression coefficient for combined centres.

Further information on the relationship between time to end-point using A.P.T. and C.O.B. was obtained from the results of a second series of experiments to be described in Part II of the report. In these experiments samples of morning milk were incubated at 18 and 22° C. from 11 a.m. on the morning of production and tested at 3 hr. intervals by A.P.T. and C.O.B.; in addition, tests were made with 50% alcohol. The results for this set of data comprising samples examined at the three centres, *K*, *L* and *M* respectively, have been similarly analysed by obtaining the regression of time to precipitation, y, with 68% and 50% alcohol on the time to C.O.B., x, in the form: $y = y_0 + b(x-30)$. The results for samples incubated at 18 and at 22° C. are given separately in Table 5.

The regression coefficients, b, from both sets of data (Tables 4 and 5) are all less than unity, but for practical purposes it is sufficient to assume that they are unity.

There are differences between centres in the time to end-point with 68 and 50% alcohol corresponding to a time to end-point of 30 hr. with the C.O.B. test. These differences between centres are comparatively trivial, bearing in mind the interval (4 hr. in the first and 3 hr. in the second series of experiments) between keeping quality observations. With

incubation at 18 or 22° C. the results indicate that in general the 50% alcohol test will reach its end-point at the same time as the C.O.B. test, and the 68% alcohol (A.P.T.) approximately 3 hr. before the C.O.B. test.

The effect of increasing the temperature of incubation from 18 to 22° C. on the time to reach the end-point will be discussed in Part II of the report.

METHYLENE-BLUE AND RESAZURIN TESTS AS MEASURES OF KEEPING QUALITY

The results of the methylene-blue and resazurin tests at 37·5 and 18° C. for the subsamples in the four sections A, B, C and D were used to study the relationship between the two dye tests, and between each dye test and the two objective measures of keeping quality—A.P.T. and C.O.B.

Relationship between times to end-point with methylene blue and resazurin

Figs. 3 and 4 show the relationship between times to reduction of methylene blue and resazurin at 18 and 37·5° C. respectively for section C subsamples; they are representative of similar diagrams for subsamples in the other three sections A, B and D. The correlation coefficient, r, for subsamples in each of the four sections is given in Table 6. The values for the two dye tests at 37·5° C. are higher than the figure for r of 0·872 obtained by Thomas, Thomas & Davies (7).

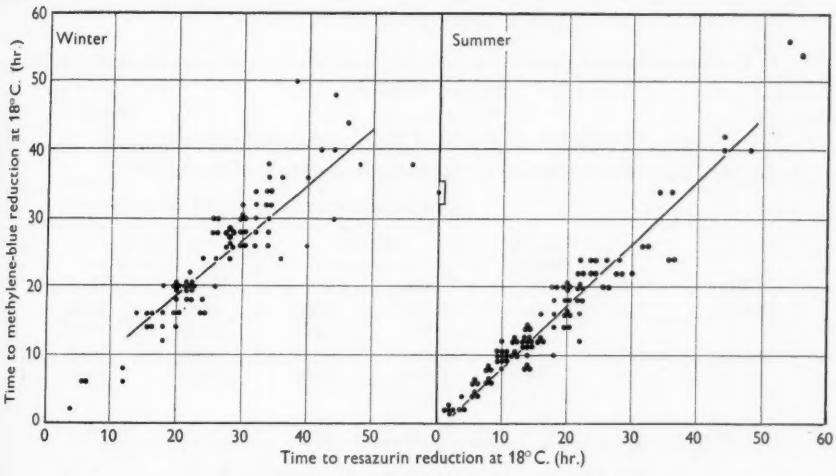

Fig. 3. Relationship between times to end-point with methylene blue and resazurin at 18° C. for section C subsamples.

Regression analysis showed the relationship between the two dye tests either at 18 or 37·5° C. to be linear and the slope unity. Therefore, a given difference in methylene-blue reduction time between two samples will mean the same difference between their resazurin reduction times, and this will apply irrespective of the time taken for reduction. In view of the high correlations (Table 6) deductions from an examination of the relationship between resazurin reduction time and any other test may for practical purposes be applied also to the relationship between methylene-blue reduction time and the same test.

Accordingly, detailed calculations have, mainly, been confined to relationships between resazurin reduction times and the results of other tests, but the conclusions to be drawn can be applied equally to the methylene-blue test.

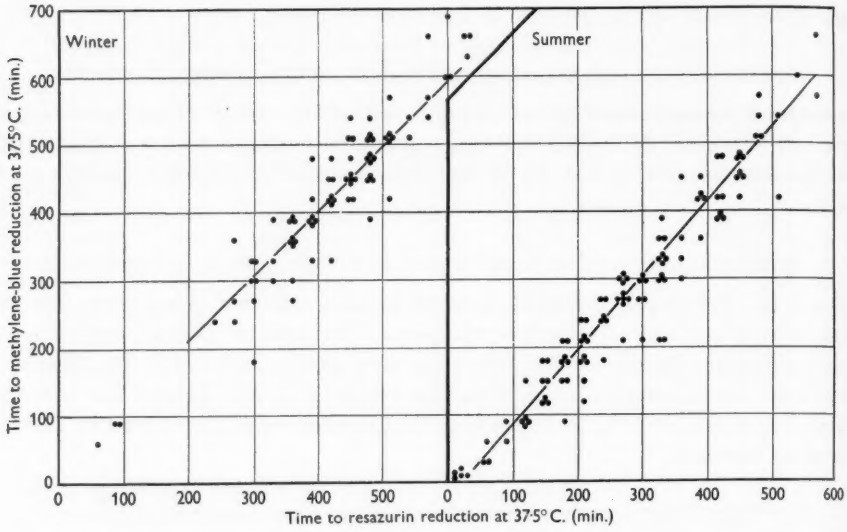

Fig. 4. Relationship between times to end-point with methylene blue and resazurin at 37·5° C. for section C subsamples.

Table 6. *Correlation coefficients r for the relationship reduction of methylene-blue/reduction of resazurin to disk* 0 *at* 37·5 *and* 18° C.

			Correlation coefficient *r* for samples incubated at			
			37·5° C.		18° C.	
Milking	Section	Age at testing (hr.)	Winter	Summer	Winter	Summer
Evening	A	19	0·956	0·926	0·948	0·891
	B	25	0·975	0·925	0·919	0·914
Morning	C	10	0·903	0·965	0·866	0·968
	D	28	0·949	0·970	0·919	0·923

Relationship between keeping quality at 18° *C. and methylene-blue and resazurin reduction times at* 18 *and* 37·5° *C.*

Figs. 5, 5A, and 6, 6A for section C subsamples are representative of the relationship between keeping quality at 18° C. and methylene-blue and resazurin reduction times at 18 and 37·5° C. respectively.

Dye tests at 18° *C.* For the relationship between the reduction time at 18° C. and A.P.T. or C.O.B., the scatter of the points, especially at the higher values, is rather wider than for that between A.P.T. and C.O.B. (Fig. 2); in other words, though reduction time is related to time to C.O.B. and could be used as an indicator of keeping quality using the C.O.B. end-point, neither reduction time is as satisfactory for that purpose as the time to A.P.T. end-point.

The time to A.P.T. or to C.O.B., however, is generally longer than either of the reduction times. For the means of the samples examined the end-point with A.P.T. and C.O.B. respectively occurred approximately 5 and 9 hr. later than resazurin.

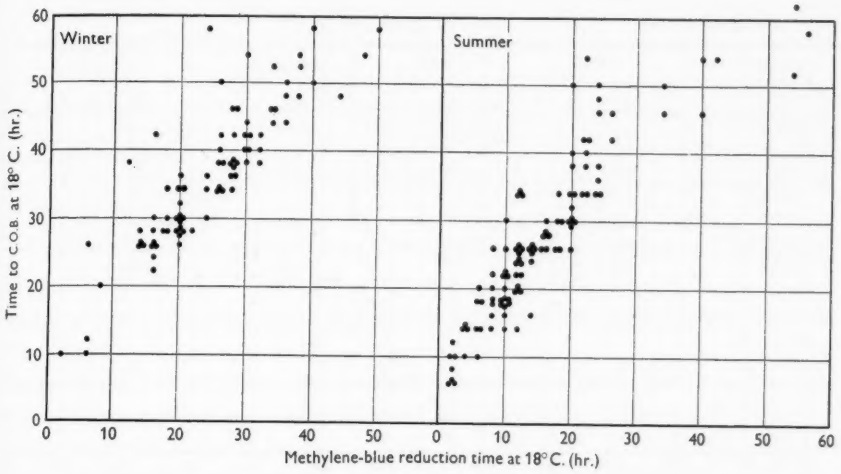

Fig. 5. Relationship between times to C.O.B. at 18° C. and to reduction of methylene blue at 18° C. for section C subsamples.

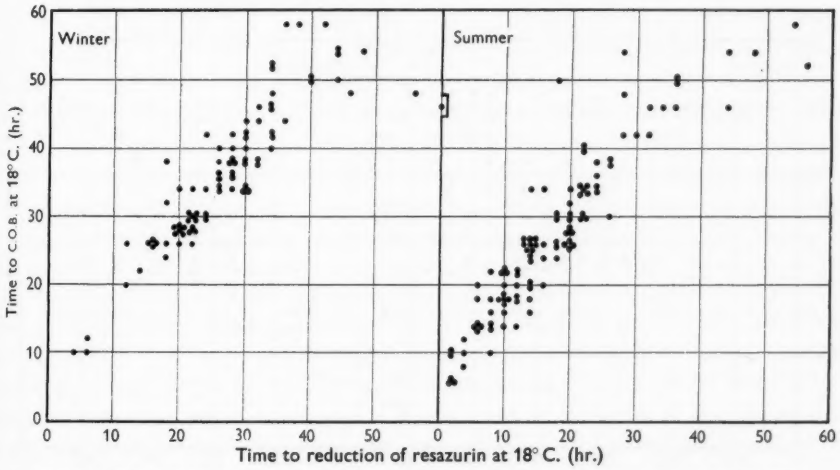

Fig. 5A. Relationship between times to C.O.B. at 18° C. and to reduction of resazurin at 18° C. for section C subsamples.

Dye tests at 37·5° C. As indicators of keeping quality there is little to choose between resazurin and methylene-blue reduction times at 37·5° C.; the scatter of the points (Figs. 6, 6A), is however, wider than for the dye tests at 18° C. (Figs. 5, 5A). A difference of 1 hr. in respect of either of the reduction times corresponds to a mean difference in the time to A.P.T. and C.O.B. of $3\frac{1}{2}$–4 hr. both in winter and in summer.

An idea of the reliability of resazurin reduction times at 18 or 37·5° C. for predicting time to A.P.T. or to C.O.B. may be obtained from Table 7, which gives the residual standard error calculated after fitting regression lines; these values give a simple measure of the scatter of the relationship.

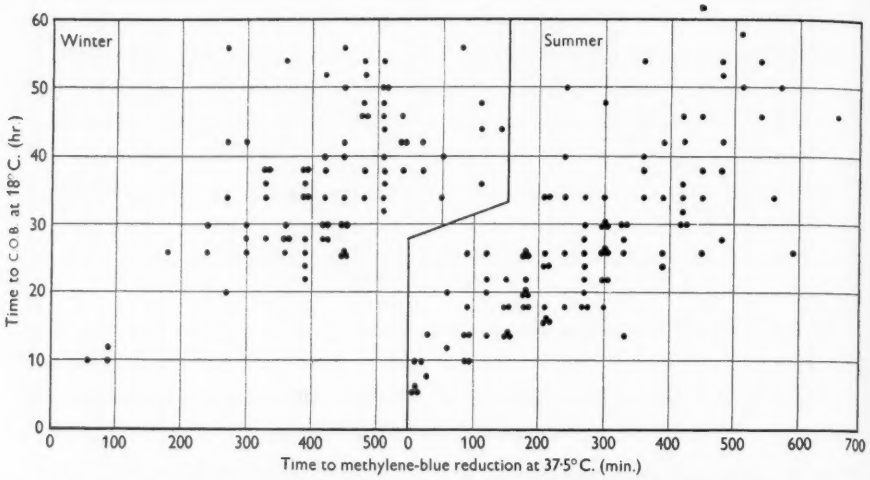

Fig. 6. Relationship between times to C.O.B. at 18 °C. and to reduction of methylene blue at 37·5° C. for section C subsamples.

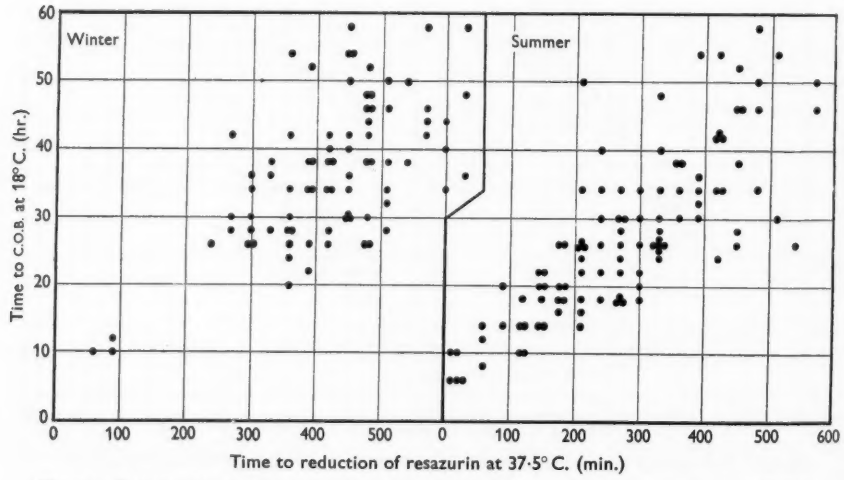

Fig. 6A. Relationship between times to C.O.B. at 18° C. and to reduction of resazurin at 37·5° C. for section C subsamples.

As an example of the interpretation of the figures in Table 7 it can be said that the A.P.T. or C.O.B. time at 18° C. of about two-thirds of a large number of samples with any given reduction time will fall within a range of 8–10 hr. (but with dye reduction times at 37·5° C. for the winter samples, the corresponding range is 15–17 hr.); similarly, one-twentieth of

Table 7. *Residual standard error (hr.) of time to* A.P.T. *and time to* C.O.B. *predicted from time to resazurin reduction to disk* 0 *at* 18° C. (R. 18) *and* 37·5° C. (R. 37·5)

		Residual standard error (hr.)			
		Evening milk		Morning milk	
		Section A (19 hr. storage)	Section B (25 hr. storage)	Section C (10 hr. storage)	Section D (28 hr. storage)
C.O.B. from R. 18:	Winter	4·59	4·76	4·98	4·84
	Summer	4·92	5·15	4·94	4·79
C.O.B. from R. 37·5:	Winter	8·39	8·42	8·07	7·72
	Summer	5·18	4·05	7·05	5·53
A.P.T. from R. 18:	Winter	4·45	5·26	5·26	4·74
	Summer	4·57	4·77	4·90	4·51
A.P.T. from R. 37·5:	Winter	8·31	8·83	7·60	7·29
	Summer	4·60	3·75	6·62	5·00

the samples will have values outside a range of 16–20 hr. (with the exception referred to, the corresponding range is 30–34 hr.).

Clearly from our results the use of dye reduction times to end-point at 18 *or* 37·5° *C. for the direct measurement of keeping quality will lead to considerable error.* Robertson & Frayer (8), and Johns (9) tasted samples, kept at 15·5° C., at 2 and 3 hr. intervals respectively and compared the results with the time to reduction of methylene blue at 37·5° C. Though it was concluded that the methylene-blue test gave a good indication of keeping quality, a study of their results shows that the scatter of the points was, in fact, as great as in our own investigations. A similar conclusion must be drawn from the results obtained by Wilson (10), who used smell to determine the end-point of keeping quality, by other workers using taste (2,11); and by Anderson & Wilson (6) who used taste and time to precipitation with 68% alcohol (A.P.T.) for measuring keeping quality.

A methylene-blue test with an incubation temperature approximating to that used for household storage was first suggested by Hiscox, Hoy, Lomax & Mattick (12) to replace the subjective tasting test at that time in general use in Britain for the routine examination of raw-milk samples (5). With thirty-one samples of high-grade milk tasted frequently, a value for r of 0·956 was obtained between time to methylene-blue reduction and to first taint, both at 15·5° C. Later, Mattick *et al.* (1) obtained an r value of 0·996 for thirty samples which had been observed at half-hourly intervals when the end-point was near, but another series of forty-nine samples involving several observers gave an r value of 0·794. Other workers (2,10,11) have not obtained quite such high correlation values, and their findings are supported by the results of our investigations.

From the work of others it is possible to speculate on the cause of the discrepancies between the results of dye reduction and keeping quality tests. Those who have studied the reducing activities of those bacteria forming a significant part of the flora of milk, have found that many, when added in pure culture to aseptically drawn milk, reduce dyes rapidly at 37·5° C. *Achromobacterium*, *Chromobacterium* and some of the aerobic spore-bearers are said to be exceptions. It has been assumed that most of the bacteria in milk participate in dye reduction. However, Wilson (10), Hobbs (13), and Rowlands & Garvie (14) have shown that, even in samples initially containing a highly heterogeneous flora, the flora at the time of reduction of dyes at 37·5 °C. is restricted to a few types, with micrococci, staphylococci and less frequently, coliform organisms and streptococci predomi-

nating. On the other hand, Wilson (10) found the predominant flora at the time of reduction of methylene blue at 17·5° C. to consist of *Achromobacterium*, *Pseudomonas fluorescens*, streptococci, micrococci and *Chromobacterium* in that order of frequency. In similar studies of the flora of milk samples at the time of the C.O.B. end-point at 22° C., Rowlands & Garvie (14) found the dominant types present to be streptococci, together with a small proportion of *Achromobacterium* and, in some samples, coliform organisms. They have shown that the streptococci found to predominate at 22° C. are, in general, different from the types isolated at the time of reduction of dyes at 37·5° C. Types of *Streptococcus lactis* or *cremoris* were frequently isolated at the time of the C.O.B. end-point, but only from occasional samples at the time of reduction of dyes at 37·5° C.; these types were not isolated by Wilson at the time of reduction of dyes at 37·5° C. Staphylococci, so commonly found to be dominant at 37·5° C., were not isolated at the time of methylene-blue reduction at 17·5° C. by Wilson, nor at the C.O.B. end-point at 22° C. by Rowlands & Garvie. Moreover, the latter authors, using a differential plating method, have shown that in samples where staphylococci predominate at the time of reduction at 37·5° C. they form only a negligible proportion of the total flora at the end-point of keeping quality at 22° C. It seems, therefore, that staphylococci on the one hand, contribute little to spoilage at normal storage temperatures, and *Str. lactis* or *cremoris* types on the other, do not play a significant part in dye reduction at 37·5° C.

Incubation at 37·5° C. leads, therefore, to enrichment of types which may contribute little to the spoilage of milk at normal household storage temperatures which keeping quality tests are designed to simulate. As stated by Wilson (10, p. 378) '...the proportions of the various types of bacteria are different in certified and churn milk before incubation, but during incubation at 37° C. the types that are most fitted to grow at this temperature rapidly gain the ascendancy over the other, usually saprophytic, types, which have a lower optimum temperature, and play therefore the main role in the reduction of the dye. So long as some of the common reducing organisms are present in moderate numbers in the original milk, natural selection can generally be depended upon to do the rest. Provided, moreover, these organisms are present in numbers more or less commensurate with the total number of organisms, the reduction time will afford a very fair index of the cleanliness of production and of the keeping quality of the milk. Both of these provisos seem to be justified in practice.' The available evidence points to the fact that in all milk samples there is a proportion of those types which become dominant during the incubation of dye tests at 37·5° C. Experience and later research both suggest that the balance between these types and those that determine keeping quality at lower temperatures is not sufficiently constant from sample to sample to ensure a sufficiently close relationship between the results of dye tests and keeping quality at lower temperatures. Wilson's statement is therefore not supported by the information now available.

The effect of temperature in encouraging enrichment of specific types may partly account for the discrepancies between dye results at 37·5° C. and keeping quality results at lower temperatures. This does not apply when tests are carried out under identical temperature conditions. Rowlands & Garvie, using pure cultures in laboratory-pasteurized aseptically drawn milk at 22° C., have found that the reducing activity of some milk bacteria does not march with their capacity to produce changes which determine keeping quality. Most bacteria which actively spoil milk at 22° C. were found to reduce dyes rapidly at the same temperature. The converse, however, was not always found to be true.

Thus, of the types so far studied, most strains of *Achromobacterium*, and some micrococci, have been found to be amongst the most active dye reducers at 22° C., but to have little effect on keeping quality. Since strains of *Achromobacterium* are commonly dominant in milk samples at the time of reduction of dyes at 17·5° C. (10), they presumably participate in dye reduction at this temperature. The initial proportions of these and similar types that are active dye reducers though in other respects relatively inert, compared with those that are, in addition, active in milk spoilage, may well determine the interval between end-points of dye reduction and keeping quality tests. If this is so we have a possible explanation of the discrepancies between dye and keeping quality tests at lower temperatures, although other factors may also be involved.

Hobbs (13) has stated '...the methylene-blue test as carried out in practice, affords a good index of bacterial metabolism in milk'. However, with some bacteria, commonly present in milk, it seems that the metabolic activities involved are not necessarily those which lead to spoilage of the type which is of greatest concern to the consumer. It may, indeed, be said to be tendencious to emphasize the capacity of the dye tests to measure bacterial metabolism, if this metabolism is a mere reflexion of the temperature of incubation during the test.

THE RELATIONSHIP BETWEEN KEEPING QUALITY AT 18° C. AND RESAZURIN DISK READINGS

The claim of resazurin to consideration as an aid to grading milk rests on the fact that intermediate stages of reduction can be observed and measured, so that results can be obtained without waiting for complete reduction. For routine purposes it would be an advantage if reliable estimates of keeping quality could be obtained in terms of resazurin disk numbers read at some specified time after setting up the tubes. The triple-hour test (15), in which samples are classified according to the particular stage of reduction in 1, 2 or 3 hr. or, as in the routine resazurin test (3) used in Britain, according to the particular stage of reduction reached in a specific time, are examples of ways in which the test is used for grading purposes.

Scatter diagrams were prepared showing the relationship between disk number at a fixed time of observation and keeping quality measured by A.P.T. or C.O.B.: Fig. 7, which shows the relationship between C.O.B. at 18° C. and resazurin disk readings after 1 hr. at 37·5° C., is representative. Inspection of the diagrams revealed, for example, that before 4 hr. at 18° C. or before 10 min. at 37·5° C. so little reduction had occurred that for almost all milk samples the disk numbers were high. Only milks of a very short keeping quality can be picked out at these times of observation. Any milks that show disk $\frac{1}{2}$ or 0 after 10 min. at 37·5° C. are likely to have very short keeping qualities: in our experiments the number of samples reducing to disk $\frac{1}{2}$ or 0 in 10 min. was too few to afford an accurate estimate of their range of keeping quality.

At later times of observation the keeping qualities of samples giving the same disk number are widely scattered. Low disk numbers usually indicate poor keeping quality milk, but the general impression from the diagrams is that, at any fixed time of observation, the difference in keeping quality between milks showing disks 3, 4 or 5 is trivial.

In Table 8 the classified results of disk readings at 18 and 37·5° C. are compared with C.O.B. At each time, 4 hr., 8 hr., etc., the samples were divided into three classes: class A those with disk readings 4–6, class B, disk 1–$3\frac{1}{2}$ and class C, disk 0 or $\frac{1}{2}$. The keeping quality times of winter milks are arbitrarily subdivided into three grades—40 hr. and over,

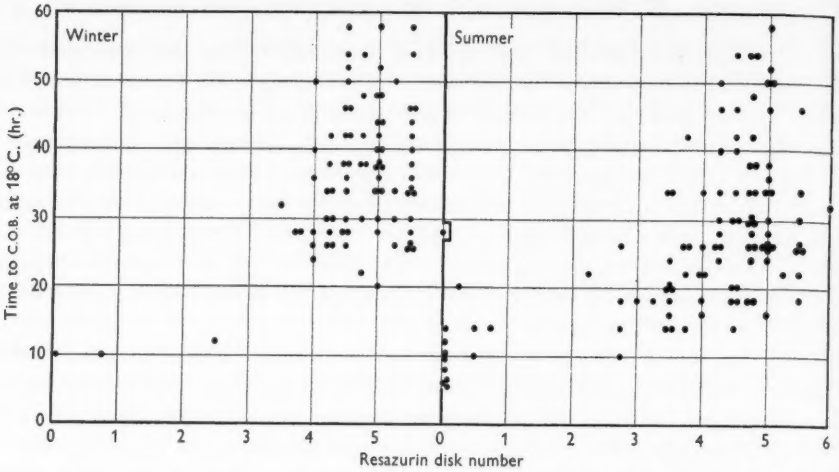

Fig. 7. Relationship between time to C.O.B. at 18° C. and resazurin disk reading after 60 min. at 37·5° C. for section C subsamples.

Table 8. *Relation for subsamples in sections B (evening milk tested at 25 hr.) and D (morning milk tested at 28 hr.) between time to* C.O.B. *at* 18° C. *and resazurin disk number at* 18 *and* 37·5° C.

Time of observation	Resazurin class*	Winter No. of samples reaching C.O.B. end-point in (hr.) 0–19	20–39	40 and over	Summer No. of samples reaching C.O.B. end-point in (hr.) 0–9	10–19	20–39	40 and over
		Resazurin test at 18° C.						
4 hr.	A	7	96	58	0	8	45	5
	B	3	11	0	0	16	19	0
	C	11	1	0	41	42	5	0
8 hr.	A	0	62	52	0	0	18	4
	B	5	33	6	0	3	29	0
	C	16	13	0	41	63	22	1
12 hr.	A	0	36	41	0	0	8	3
	B	0	38	17	0	0	15	1
	C	21	34	0	41	66	46	1
16 hr.	A	0	16	30	0	0	4	2
	B	0	35	27	0	0	8	2
	C	21	57	1	41	66	57	1
		Resazurin test at 37·5° C.						
10 min.	A	—	—	—	1	54	69	5
	B	—	—	—	18	11	0	0
	C	—	—	—	22	1	0	0
30 min.	A	—	—	—	0	28	61	5
	B	—	—	—	1	23	8	0
	C	—	—	—	40	15	0	0
60 min.	A	8	93	57	0	11	41	5
	B	5	14	1	0	19	24	0
	C	8	1	0	41	36	4	0
90 min.	A	1	83	53	0	2	30	5
	B	9	22	5	0	18	31	0
	C	11	3	0	41	46	8	0
120 min.	A	1	64	44	—	—	—	—
	B	9	37	14	—	—	—	—
	C	11	7	0	—	—	—	—
180 min.	A	0	34	34	—	—	—	—
	B	7	56	24	—	—	—	—
	C	14	18	0	—	—	—	—

* Class A equals disk 4–6; B disk 1–3½; C disk ½ or 0.

20–39 hr. and under 20 hr.; for the summer milks four grades are used—40 hr. and over, 20–39 hr., 10–19 hr. and under 10 hr. The results for morning and evening milks were so similar that they have been combined. Only results for subsamples in section B (p.m. 25 hr. storage) and section D (a.m. 28 hr. storage) are reproduced, as those for subsamples in sections A and C were similar.

The table shows that disk readings do not provide a satisfactory basis for discriminating between milks of different keeping quality. For example, in winter most of the samples placed in class C by the resazurin test after 8 hr. at 18° C. reached the end-point of the C.O.B. test in less than 20 hr., but errors would be committed by assuming all samples in this class to be of the lowest grade. Moreover, class A samples, though more frequently of longer keeping quality than class B samples, are by no means invariably so. Even at 16 hr. when about two-thirds of the samples in class A have a C.O.B. time in excess of 40 hr., almost one-half of the samples in class B also reach this standard, and many errors would be committed if resazurin classes were made a basis for price penalties or bonuses. Tests at 37·5° C. are equally unsatisfactory if not more so. For example, at 90 min. most of the class C samples have a keeping quality of under 20 hr., but other samples of equally low keeping quality are found amongst those in classes A and B.

TEMPERATURE COMPENSATION AND THE RESAZURIN TEST

The routine resazurin test (3) embodies a temperature compensation scale. Samples are tested when approximately 24–28 hr. old. The time of incubation at 37·5° C. is varied according to the mean of the shade atmospheric temperature for the period between production and testing; thus when the mean shade atmospheric temperature is over 60° F. the disk reading is taken after 15 min., and when it is 40° F. or under, after 120 min. The present work has shown the general unreliability of resazurin disk readings at fixed times as measures of keeping quality. It was therefore felt that no good purpose would be served by an examination of the reliability of the existing temperature compensation scale.

THE RELATIONSHIP OF THE RESAZURIN DISK NUMBER AND TIME OF OBSERVATION

If the disk number of a sample is recorded at frequent intervals it is possible to prepare a graph relating time and disk number. This was done for a large number of samples, and it was found that the shape of the time/disk curve varied widely from sample to sample. Thus of samples reducing to disk 0 in about 6 hr. at 37·5° C., some might give disk 4 or higher at 2 hr., others disk 2, and others intermediate values. Examples of time/disk curves are given in Fig. 8, readings being taken every 15 min. If the shape of the time/disk curve were constant the accuracy of prediction of keeping quality by disk number at any specified time would be improved, since it would be of the same order of accuracy as prediction from the end-point of reduction.

The shape of the time/disk curve is influenced, to some extent, by the number of leucocytes present in the milk. Cell counts, using the Breed method (16), were made on all the samples, and a statistical test was made of the relationship between the departure from linearity of the time/disk curve and the cell count. The effect of cells, though small, is undoubtedly significant. Milks with high cell counts tend to give time/disk curves which are concave upwards; high bacterial populations also appear to influence the curve in the same direction; the cell count and the plate count are themselves unrelated. A detailed

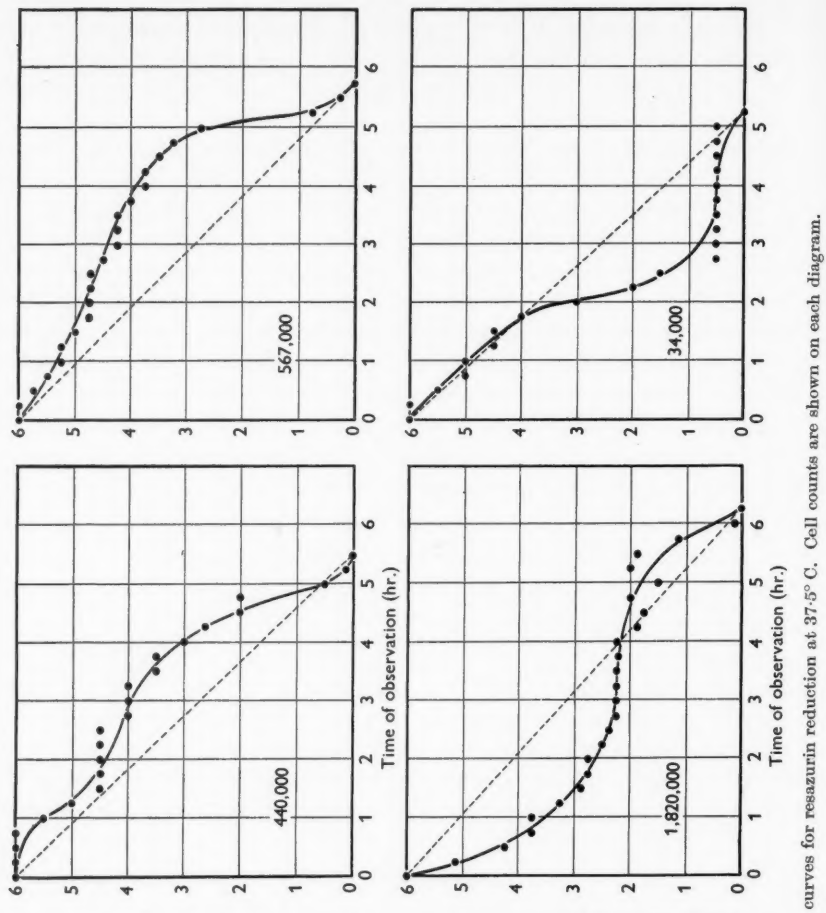

Fig. 8. Examples of time/disk curves for resazurin reduction at 37·5° C. Cell counts are shown on each diagram.

examination of the records has been made, but there is no indication that even within a limited range of cell counts or of plate counts the relationship between disk number and keeping quality is more satisfactory.

Other workers (17,18,19,20,21,22,23,24,25,26) have observed that large numbers of cells in milk may cause rapid partial reduction of resazurin, the effect being more pronounced in fresh milks than in milks tested after ageing for 24 hr.

Our results show that the shape of the resazurin disk curve is not solely determined by the cell content or by the total count of bacteria in the milk. Others (27,28,29), on the basis of electrometric measurements, have emphasized the complexity of the factors responsible for the first stage of reduction of resazurin (blue, disk 6) to resorufin (pink, disk 1); as shown by Twigg (30), this stage is irreversible. Accordingly, at any particular time of observation the colour changes represented by the disk numbers do not necessarily give an indication of the oxidation-reduction potential (E_h) of the system. Any reducing substance of weak intensity may at any particular moment cause a temporary negative drift in E_h which will be recorded as a lower disk number in the resazurin test, and this colour will be retained even though the system may be subsequently reoxidized. In the presence of cells, for example, resazurin and methylene blue may be partially reduced, but on inversion of the sample tube the system may be quickly reoxidized; methylene blue, being reversible, will regain its full colour, but the resazurin disk number will continue to record the temporary potential drift. The variable nature, in different milks, of the factors other than bacteria causing slight temporary or even permanent negative drift in the E_h complicates the interpretation of resazurin disk numbers at any specified time of observation. The use of disk numbers cannot be regarded as a satisfactory basis for assessment of the keeping quality of milk.

ESTIMATION OF KEEPING QUALITY BY COLONY COUNTS

In the present series of experiments plate counts were made at 22, 30 and 37·5° C. So far only a preliminary examination of the results has been made, but a large number of scatter diagrams have been prepared showing the relationship between the logarithm of the counts at the different temperatures and A.P.T. and C.O.B. at 18° C. The scatter of the points was too great to justify the use of the colony count as a measure of keeping quality. The scatter appeared to be a little greater for the count at 37·5° C. than for either of the other two temperatures, and this count is probably the least good for predicting keeping quality. The logarithms of the plate counts are less highly correlated with keeping quality than the dye reduction times, with the possible exception of the 37·5° C. reduction times in winter. Doubling the 30° C. plate count (i.e. an increase of 0·30 in the logarithm of the count) corresponds to about a 2½ hr. reduction in A.P.T. time at 18° C. in winter, and in summer to about 2 hr. Though the corresponding calculations for the plate counts at 22 and 37·5° C. have not been made, the diagrams show that the magnitude of the effect is about the same.

TITRATABLE ACIDITY AS A MEASURE OF KEEPING QUALITY

The shortcomings of the acidity test are well known. Nevertheless, it has been widely used by buyers as an objective measure of the suitability of milk for acceptance. Some standard is set, e.g. 0·18% lactic acid, which when exceeded is taken as sufficient confirmatory evidence for rejection of a consignment. It may be seen from the figures in Table 9 that whatever may be the mean value of the acidity results for any series of samples either

Table 9. *Frequency distribution for section C subsamples according to titratable acidity values initially, at time of first taint (T), at end-point with 68 % alcohol (A.P.T.) and clot-on-boiling (C.O.B.)*

	Time of testing															
	*Initially Centre				At time of first taint Centre				At time of A.P.T. end-point Centre				At time of C.O.B. end-point Centre			
Lactic acid (%)	*K*	*L*	*M*	All	*K*	*L*	*M*	All	*K*	*L*	*M*	All	*K*	*L*	*M*	All
								Winter samples								
Less than 0·128	2	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.
0·128–0·137	6	.	1	7	.	.	.	.	.	.	.	.	.	.	.	.
0·138–0·147	12	1	2	15	1	.	.	1	.	.	.	.	.	.	.	.
0·148–0·157	5	4	1	10	.	.	.	.	.	.	.	.	.	.	.	.
0·158–0·167	4	7	7	18	2	.	.	2	1	.	1	2	.	.	.	.
0·168–0·177	1	11	8	20	3	1	1	5	.	1	.	1	1	.	.	1
0·178–0·187	.	6	2	8	.	3	4	7	2	.	5	7	1	.	.	1
0·188–0·197	.	1	2	3	5	1	5	11	7	3	4	14	1	.	.	1
0·198–0·207	.	1	1	2	3	3	3	9	5	5	5	15	4	.	2	6
0·208–0·217	.	1	.	1	1	.	4	5	1	1	3	5	6	.	5	11
0·218–0·227	.	.	.	.	.	6	2	8	1	4	2	7	2	3	5	10
0·228–0·237	.	.	.	.	.	2	2	4	.	6	1	7	2	2	2	6
0·238–0·247	.	.	.	.	.	3	1	4	1	1	1	3	7	2	4	13
0·248–0·257	.	.	.	.	.	1	.	1	.	4	.	4	2	2	1	5
0·258–0·267	.	.	.	.	2	2	.	4	.	3	1	4	1	4	1	6
0·268–0·277	.	.	.	.	.	1	.	1	.	1	.	1	1	2	1	4
0·278–0·287	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	2
0·288–0·297	.	.	.	.	.	1	.	1	.	.	.	.	.	3	2	5
0·298–0·307	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	2
0·308–0·317	.	.	.	.	.	1	.	1	.	.	.	.	.	2	.	2
0·318–0·327	.	.	.	.	.	.	.	.	.	.	.	.	.	3	.	3
0·328 and over	.	.	.	.	.	.	.	.	.	.	.	.	.	3	.	3
No. of samples	30	32	24	86	17	25	22	64	18	29	23	70	28	30	23	81
Mean	0·145	0·170	0·170	0·160	0·195	0·225	0·205	0·210	0·200	0·225	0·205	0·210	0·225	0·280	0·235	0·245
								Summer samples								
Less than 0·128	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
0·128–0·137	6	.	.	6	.	.	.	.	.	.	.	.	.	.	.	.
0·138–0·147	7	.	3	10	.	.	.	.	.	.	.	.	.	.	.	.
0·148–0·157	12	1	10	23	.	.	1	1	1	.	.	1	.	.	.	.
0·158–0·167	6	4	12	22	4	.	1	5	2	.	.	2	.	.	.	.
0·168–0·177	2	12	6	20	1	.	2	3	2	.	1	3	1	.	.	1
0·178–0·187	1	3	3	7	3	.	8	11	6	.	7	13	.	.	.	.
0·188–0·197	1	9	1	11	4	.	4	8	3	.	6	9	.	.	.	.
0·198–0·207	.	5	.	5	.	.	8	8	4	.	7	11	1	.	1	2
0·208–0·217	.	1	1	2	1	.	5	6	3	.	5	8	3	.	4	7
0·218–0·227	.	.	.	.	2	.	1	3	4	.	1	5	6	.	3	9
0·228–0·237	.	.	.	.	1	2	5	8	2	.	3	5	9	.	5	14
0·238–0·247	.	.	.	.	1	2	1	4	1	3	.	4	5	1	6	12
0·248–0·257	.	.	.	.	1	.	.	1	.	1	.	1	2	.	7	9
0·258–0·267	.	.	.	.	3	1	.	4	.	1	.	1	3	1	5	9
0·268–0·277	.	.	.	.	1	2	.	3	.	1	.	1	2	8	3	13
0·278–0·287	.	.	.	.	.	3	.	3	.	.	.	.	1	4	.	5
0·288–0·297	.	.	.	.	.	.	.	.	.	.	.	.	1	3	1	5
0·298–0·307	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	3
0·308–0·317	.	.	.	.	.	1	.	1	.	.	.	.	.	2	.	2
0·318–0·327	.	.	.	.	.	1	.	1	.	.	.	.	.	.	.	.
0·328 and over	.	.	.	.	1	3	.	4	.	.	.	.	.	4	.	4
No. of samples	35	35	36	106	23	15	36	74	28	6	30	64	34	25	36	95
Mean	0·150	0·180	0·165	0·165	0·215	0·285	0·200	0·220	0·200	0·250	0·200	0·205	0·235	0·290	0·245	0·255

* Initial acidity determinations made when samples were 10 hr. old; samples stored at 18° C. and subsequent titrations made at 4 hr. intervals.

initially or at the time of the end-point of the different measures of keeping quality, there is a wide spread of values. It would be impossible to take as the end-point any fixed acidity value without including many samples that are unsatisfactory or excluding many that are satisfactory. With the approach of the end-point of the keeping quality tests there is generally a rapid rise in acidity, and with observations made only at 4 hr. intervals

this may partly account for the spread of the acidity values at these end-points. It cannot, however, account for the spread of the results for acidity determinations early in the life of the milk. Acidities tend to increase in the centre order *K*, *M*, *L*, but there is no ready explanation for the variations in acidities from centre to centre. It is not possible to say whether these inter-centre differences are genuine, due to local variations in the composition of the milk, or to unintentional differences in the assessment of the end-point of the titration.

SUMMARY AND CONCLUSIONS

1. The main object of the experiments described in this part of the report was to examine the suitability of dye tests as measures of the keeping quality of raw milk. The data were obtained from the examination of samples of farm milk supplies taken throughout the year in three widely separated areas of England and Wales. Before examination the morning and evening milks respectively were stored at shade atmospheric temperature for 10 and 19 hr. as prescribed for designated milk in England and Wales (4) and for 28 and 25 hr. as prescribed for the routine resazurin test of the National Milk Testing and Advisory Scheme (3). After storage the following tests were applied: taste (*T*), precipitation with 68% alcohol (A.P.T.), clot-on-boiling (C.O.B.) and acidity, all at 4 hr. intervals on portions stored at 18° C.; methylene-blue and resazurin tests at 18° C. with observations at 2 hr. intervals, and also at 37·5° C. with observations at ½ hr. intervals; colony count on yeastrel milk agar at 22, 30 and 37·5° C.

2. In general there were no major differences between centres, between winter and summer, between morning and evening milk, or between the results after storage for the different periods before beginning the tests. Accordingly, most of the tables and diagrams reproduced refer to morning samples, from all centres, examined after storage for 10 hr.; notable differences which occurred are referred to in the text.

3. Keeping quality measured by the time to the first record of taint (*T*) is inadmissible as a basis for assessing the value of other bacteriological tests; taste is too subjective and some taints are not bacterial in origin. The evidence presented shows that the results for A.P.T. and C.O.B. are highly correlated, and either method is suitable as a measure of the end-point of keeping quality. With incubation at 18 or 22° C. the end-point using A.P.T. occurs earlier than C.O.B.; the difference on the average is about 3 hr. Though in a high proportion of samples the end-points using *T* and A.P.T. coincide or occur within two successive observations (an interval of 4 hr. in these investigations), A.P.T. cannot entirely replace taste in the day to day commercial contacts of producer, distributor and consumer.

4. At 18 or 37·5° C. the times to end-point of the resazurin and methylene-blue tests are highly correlated. A given difference in methylene-blue reduction time between two samples will mean about the same difference between their resazurin reduction times, and this is independent of the time to reduction. Accordingly, any conclusions about the relationship between time to reduction of resazurin and any other test will apply also to the relationship between methylene-blue reduction time and the same test.

5. There is little to choose between the time to reduction at 37·5° C. of methylene blue or resazurin as measures of keeping quality. Their reliability for predicting keeping quality however is poor, particularly in winter. Our results do not support the use of dye tests at this temperature as measures of keeping quality.

The above remarks also apply to dye tests at 18° C., though the scatter of the relationship between dye and keeping quality tests at this temperature is less than for the dye tests at 37·5° C. The time at 18° C. to A.P.T. or to C.O.B. is generally longer than the time to reduction either of methylene blue or of resazurin at the same temperature; for the means of the samples examined in our experiments, the end-point with A.P.T. and C.O.B. respectively occurs approximately 5 and 9 hr. later than resazurin.

6. Other workers have shown that in practically all milk samples the types of bacteria predominant at the time of dye reduction at 37·5° C. and at the end-point of keeping quality at 18–22° C. are different. This factor alone may adequately explain the discrepancies which occur between dye reduction and keeping quality results.

At 18–22° C. many milk samples contain types of bacteria that reduce dyes actively but have no apparent effect on keeping quality. In different milk samples variations in the proportion of these types relative to those that are active in milk spoilage may well explain some of the discrepancies between dye and keeping quality results at these lower temperatures.

7. Resazurin disk readings at a fixed time of observation are not satisfactory for discriminating different levels of keeping quality. Practically all samples placed in class C (disk $\frac{1}{2}$ or 0) in the resazurin test would be of poor keeping quality, but some samples with equally poor keeping qualities would be placed in class B (disk 1–3$\frac{1}{2}$) or even in class A (disk 4–6). The shape of the time/disk curve is found to vary widely from one sample to another. High leucocyte counts and high colony counts appear to have a similar influence on the shape of the time/disk curve, but these factors alone are not adequate to explain the variations observed with different samples. The irreversibility of the first stage of reduction of resazurin to resorufin (disk 6 to 1) may partly account for the variations in the shape of the time/disk curve.

8. Neither acidity, nor colony counts at 22, 30 or 37·5° C. are suitable measures of the keeping quality of milk.

9. Any of the bacteriological tests discussed will provide, on the average, an approximate estimate of keeping quality. In the routine grading of milk supplies, however, we are dealing with individual samples from individual producers, and for this purpose accurate prediction of keeping quality from the results of other tests is not possible. To determine the keeping quality of milk a direct method of assessment must be used; to deduce it from observations made by other means is not justified.

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APPENDIX 1. REPRODUCIBILITY OF KEEPING QUALITY AND DYE TESTS

Reproducibility of the clot-on-boiling (C.O.B.) *end-point*

Tests on seventy samples were carried out at the two centres *L* and *M*. From each sample of milk five subsamples each consisting of approximately 60 ml. in a 3 oz. sample bottle (B.S.S. 809, 1938) were placed in a $22 \pm 1°$ C. water-bath and examined at 3 hr. intervals. The first recorded + if preceded by − or . was taken as the end-point; when a − reading preceded a + + the end-point was taken as 1 hr. earlier than the + +; when a . reading preceded + + the end-point was taken as 2 hr. earlier than the + +. The results which were similar at the two centres are given in Appendix Table 1.

Appendix Table 1. *Frequency distribution of range of times to* C.O.B. *end-point in quintuplicate tests, at* 22° C., *observed every* 3 *hr.* (*centres L and M*)

Mean C.O.B. time in range* (hr.)	Difference between earliest and latest C.O.B. times (hr.)							No. of samples
	0	1	2	3	4	5	6	
16–25	9	1	0	0	0	0	0	10
26–35	28	1	2	2	0	0	0	33
36–45	7	3	2	4	0	2	3	21
46–55	5	0	1	0	0	0	0	6
Total	49	5	5	6	0	2	3	70

* Samples 24 hr. old when incubation at 22° C. began.

The end-point of the five subsamples occurred at the same observation time with 70%, and within two successive 3 hr. observations with 93% of the samples. For the remaining 7% the end-points of the five subsamples occurred within three successive 3 hr. observations. The larger discrepancies occurred with milks having the longer mean times to C.O.B.

end-point. Investigations of the reproducibility of the alcohol precipitation test, and of dye reduction tests (see below) showed a similar trend.

These results indicate that the C.O.B. test taken as end-point is highly reproducible, and the test may, in this respect, be regarded as a satisfactory objective method of measuring the keeping quality of raw milk.

Rate of change to end-point. To determine the rate of change from negative to positive C.O.B. a series of fifty samples was examined at 15 min. intervals. In these tests even the slightest precipitate was recorded +. It was found that results could be classed as negative − or + in most instances. With forty-seven of the samples the change from − to + took place within 15 min.: with the remaining three samples the interval was 30 min., a . being recorded 15 min. before the + end-point.

Reproducibility of the time to end-point with 68% *alcohol* (A.P.T.)

Tests on eighty-eight samples were carried out at the three centres *K*, *L* and *M*. The procedure was the same as in the C.O.B. reproducibility experiment except that the samples were incubated at 18 ± 1° C. and observations were made at 4 hr. intervals. The first recorded + if preceded by − or . was taken as the end-point; when a − reading preceded a + + the end-point was taken as midway between the two readings; when a . reading preceded + + the end-point was taken at the time of recording .. There was no apparent difference between centres, and the results are combined in Appendix Table 2.

Appendix Table 2. *Frequency distribution of range of times to* A.P.T. *end-point in quintuplicate tests at* 18° C. *observed every* 4 *hr.* (*centres K, L and M*)

Mean A.P.T. time in range* (hr.)	Difference between earliest and latest A.P.T. time (hr.) 0	2	4	6	8	No. of samples
0–20	12	2	0	0	0	14
21–40	20	4	5	0	0	29
41–60	29	9	3	0	1	42
61–80	1	0	0	0	2	3
Total	62	15	8	0	3	88

* Samples 24 hr. old when incubation at 18° C. began.

The end-point of the five subsamples occurred at the same time with 70%, and within two successive 4 hr. observations with 97% of the samples. The similarity of the results to those obtained with the C.O.B. test may be observed, and the same conclusions may be drawn regarding the suitability of A.P.T. using 68% alcohol as a measure of the end-point of keeping quality of raw milk.

Reproducibility of the methylene-blue and resazurin tests at 37·5° C.

From samples of a.m. milk about 28 hr. old, five tubes were set up for each of the dye tests, the tubes then being incubated at 37–38° C. and read at 15 min. intervals until reduction was complete. Approximately equal numbers of samples were tested at each of the three centres *K*, *L* and *M*. Examination of the data showed that there was no difference in the reproducibility of the results at the different centres.

Appendix Table 3. *Frequency distribution of range of times to complete reduction of dyes in quintuplicate tests at 37·5° C. (centres K, L and M)*

Mean time to complete reduction (min.)	Difference between earliest and latest reduction time (min.)							Total samples
	0	15	30	45	60	75	90 and over	
				Methylene blue				
0–120	11	0	0	0	0	0	0	11
121–240	11	0	0	1	0	0	0	12
241–360	17	6	1	1	0	0	0	25
361–480	19	12	2	1	0	1	1	36
481–600	12	9	5	1	0	0	0	27
Over 600	2	1	0	1	0	2	1	7
Total	72	28	8	5	0	3	2	118
				Resazurin				
0–120	8	2	0	0	0	0	0	10
121–240	8	1	1	0	0	0	0	10
241–360	14	10	1	1	0	0	0	26
361–480	23	13	3	1	1	0	1	42
481–600	5	10	7	2	2	0	1	27
Over 600	0	3	0	0	0	0	0	3
Total	58	39	12	4	3	0	2	118

Appendix Table 3 shows the distribution of the range of times to complete reduction of methylene blue and resazurin for the 118 samples examined. That the agreement between readings of replicate tubes is close can be seen without detailed analysis of the results of this experiment. In about 85% of samples the five tubes for either dye were reduced in the same or in two successive 15 min. observations—a very satisfactory agreement. As with C.O.B. (Appendix Table 1) and A.P.T. (Appendix Table 2) the error appears to be greater with longer reduction times. For the methylene-blue test it has been observed (1) that with increasing reduction time the actual error increases but the percentage error decreases.

The agreement between resazurin disk readings for the sets of five tubes at any fixed time is even closer; Appendix Table 4 shows no important discrepancy between disk readings of replicate tubes.

Appendix Table 4. *Frequency distribution of range of resazurin disk readings of quintuplicate tubes at fixed times of observation (centres K, L and M)*

Time observed (min.)	Difference between highest and lowest disk reading			Samples completely reduced prior to fixed time	Total samples
	0	¼ or ½	1½		
60	101	13	1	3	118
90	96	14	0	8	118
120	99	10	0	9	118

The results in Appendix Tables 3 and 4 confirm the findings of others (1,2,3), that for neither test is reproducibility likely to be a factor limiting its practical utility, and for raw milk a single tube per sample should be sufficient for routine purposes.

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APPENDIX 2. FURTHER OBSERVATIONS ON THE ALCOHOL TEST

The effect of varying the concentration of alcohol

From the results given in Table 5 (p. 168) it may be seen that, in general, precipitation with 50% alcohol and C.O.B. occur almost simultaneously; with 68% alcohol precipitation occurs approximately 3 hr. before C.O.B. For some purposes, e.g. in testing the suitability of milk for acceptance at the creamery platform, it might be advantageous to obtain an even earlier prediction of the C.O.B. end-point than is provided by the use of 68% alcohol. Accordingly, it was decided to investigate this aspect of the alcohol test.

Observations were made on twenty-one samples of raw milk received for other routine tests at centre *K* on 31 January 1946. Prior to starting the tests the samples had been stored from the time of milking for approximately 24 hr. at a mean shade atmospheric temperature of 46° F. Owing to the low temperature of storage extensive bacterial deterioration was unlikely to have occurred. The times were recorded, with incubation at $18 \pm 1°$ C., for each of the samples to reach C.O.B. and to precipitate with alcohol ranging in concentration from 80 to 30%. At the time of transfer to the 18° C. bath ten of the samples precipitated with 80% alcohol, five with 76%, one with 72% and one with 68%; for the lower concentrations precipitation frequently occurred after C.O.B., and it was not always possible to continue testing until complete results were obtained. The results are presented graphically in Appendix Fig. 1, and the main conclusions are clear from the graphs.

Appendix Table 5. *Effect of varying the concentration of alcohol on the relationship between time to precipitation with alcohol (y) and to* C.O.B. *(x)*

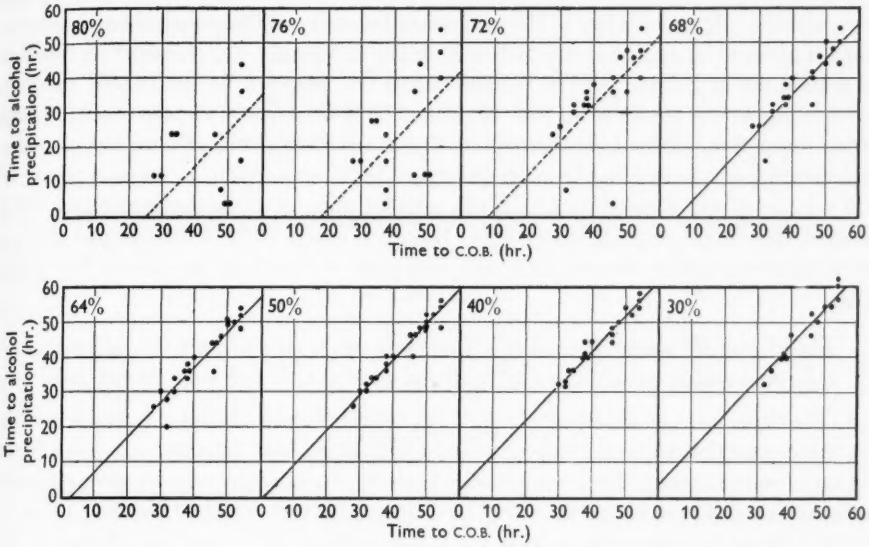
Alcohol (%)	No. of samples	Slope of regression line (b)	Residual standard error (s)	Time to precipitation with alcohol (y_0) corresponding to C.O.B. time of 42 hr.
80	11	$0{\cdot}27 \pm 0{\cdot}41$	13·29	$18{\cdot}41 \pm 4{\cdot}08$
76	16	$0{\cdot}94 \pm 0{\cdot}40$	13·54	$24{\cdot}41 \pm 3{\cdot}39$
72	20	$0{\cdot}91 \pm 0{\cdot}27$	10·01	$34{\cdot}05 \pm 2{\cdot}24$
68	20	$1{\cdot}02 \pm 0{\cdot}12$	4·47	$37{\cdot}39 \pm 1{\cdot}00$
64	21	$1{\cdot}05 \pm 0{\cdot}09$	3·30	$39{\cdot}33 \pm 0{\cdot}72$
50	20	$1{\cdot}00 \pm 0{\cdot}06$	2·21	$41{\cdot}40 \pm 0{\cdot}49$
40	19	$0{\cdot}99 \pm 0{\cdot}06$	1·94	$43{\cdot}79 \pm 0{\cdot}45$
30	14	$1{\cdot}15 \pm 0{\cdot}08$	2·25	$44{\cdot}77 \pm 0{\cdot}64$

Appendix Table 5 gives the values of y_0 and b with their standard errors for the relationship $y = y_0 + b(x - 42)$*—the regression line calculated from the available data—where y is the time to precipitation with alcohol and x the time to C.O.B. The table also contains the residual standard error s which is a convenient measure of the spread of the relationship.

With the exception of that for 80% alcohol, the slopes b are all approximately unity. With 80% alcohol, however, the slope is badly determined and has a large proportionate error ($0{\cdot}27 \pm 0{\cdot}41$), but on investigation it proved not to differ significantly from unity. Taking the slope to be unity in all cases, a new series of values for y_0, corresponding to values of $x = 42$, and s are obtained. The differences between these new values for time to precipitation with alcohol and time to C.O.B., i.e. $y_0 - 42$, together with the new residual standard errors s, are given in Appendix Table 6. The lines on the graphs in Appendix Fig. 1 satisfy these new conditions, i.e. they represent the equation $y = y_0 + b(x - 42)$ when $b = 1$, and their interceptions on the y axis equal $y_0 - 42$, the values given in Appendix Table 6.

* See footnote, p. 167.

Both the results in Appendix Table 6 and the graphs in Appendix Fig. 1 indicate that close relationships exist between the concentration of alcohol and, first, the difference between times to precipitation and to C.O.B. ($y_0 - 42$ in Appendix Table 6); and, second, the variability of the times to precipitation compared with the constant times to C.O.B. (s in Appendix Table 6). It is clear that while the use of a higher concentration of alcohol enables the end-point of the test to be reached more quickly, the test is then also subject to



Appendix Fig. 1. Relationships between the time to C.O.B. and the time to precipitation with alcohol of different concentrations.

Appendix Table 6. *Difference between times to precipitation with varying strengths of alcohol and to* C.O.B., *and the residual errors* (s) *of the alcohol precipitation times*

Alcohol (%)	No. of samples	Time to precipitation with alcohol—corresponding time to C.O.B. ($y_0 - 42$)	Residual standard error (s)
80	11	−24·91±4·41	14·63
76	16	−17·63±3·27	13·09
72	20	− 8·00±2·19	9·78
68	20	− 4·60±0·97	4·36
64	21	− 2·67±0·71	3·25
50	20	− 0·60±0·48	2·15
40	19	1·79±0·43	1·89
30	14	3·14±0·65	2·45

other factors which introduce great variability. This variability is at a minimum for some concentration of alcohol between 30 and 50%, and the residual standard error is then less than half its value when 68% alcohol is used. For concentrations of alcohol in excess of 68% results are not reliable as measures of the keeping quality of milk. This need not, however, preclude the use of higher concentrations for the preliminary examination of suspected consignments on the dairy platform, the results being confirmed by other methods.

Because of the rapidity with which alcohol tests can be applied the use of 72% or even 76% alcohol might prove advantageous in practice for the selection of individual consignments of doubtful keeping quality. Dahlberg & Garner (1) advocated 75% alcohol for the detection of raw milk liable to cause heat instability of evaporated milk. The use of 68, 72 and 76% alcohol for the detection of unsatisfactory raw milk at the creamery platform has recently been investigated by Hughes & Ellison (2). Of 378 samples tested, approximately 33% precipitated with 76% alcohol, 27% with 72% alcohol, and 23% with 68% alcohol. However, some of the samples failing to precipitate with these concentrations of alcohol reduced resazurin to disk 3½ or less in 10 min.—the standard for rejection in general use in Britain. Of the samples failing this standard by the 10 min. resazurin test the proportion not detected with 76, 72 and 68% alcohol was respectively 11, 16 and 20%. Jones-Evans (3) used 72% alcohol for the examination of milk arriving at creameries, and determined the residual keeping quality at 20° C. with C.O.B. as the end-point. Of 219 samples giving a precipitate with this concentration of alcohol approximately 93% reached the C.O.B. end-point within 6 hr. However, nearly 20% of 519 samples giving a negative alcohol result also reached C.O.B. end-point within 6 hr., and about 7% within 3 hr.

The effect of the alcohol instability of fresh milk on the time to reach the end-point of keeping quality using 68 *and* 50% *alcohol and* C.O.B. *to determine the end-point*

In the experiment on the effect of varying the concentration of alcohol it was found that the relationship between time to precipitation with alcohol and time to C.O.B. was affected by the concentration of alcohol (Appendix Table 6). Some of the samples gave a precipitate with alcohol concentrations of 68% or higher when first tested. However, Appendix Table 7 shows that the time to reach C.O.B. end-point at 18° C. was not affected by the instability to alcohol at the first test. Although giving a precipitate with alcohol at concentrations ranging from 68 to 80%, these samples took 28 hr. or more at 18° C. to reach the C.O.B. end-point.

Appendix Table 7. *Range of times to* C.O.B. *end-point for samples stable or unstable to alcohol of different concentrations when first tested after* 24 *hr. storage at a mean shade temperature of* 46° F.*

Concentration of alcohol used (%)	Unstable to alcohol at first test	Range of times to C.O.B. at 18° C. (hr.)	Not unstable to alcohol at first test	Range of times to C.O.B. at 18° C. (hr.)
64	0	—	21	28–54
68	1	32	20	28–54
72	1	32	20	28–54
76	5	32–52	16	28–54
80	10	32–52	11	28–54

* 21 samples examined.

To obtain further information about the possible relationship between the alcohol instability of fresh milk and the time to reach the C.O.B. end-point a further experiment was carried out at centre *K*. In this experiment individual quarter-samples drawn aseptically from cows in the same herd were stored in a refrigerator overnight. Between 9 and 10 a.m. the following morning the milk of each cow was bulked separately, and tested with alcohol ranging in concentration from 95 to 68%. Duplicate portions o each sample

were then inoculated with an 18 hr. culture of an active strain of *Streptococcus lactis*, grown in sterile milk at 30° C. The samples were incubated in a water-bath at 18 ± 1° C. and tested at 4 hr. intervals until the end-point was reached with 68 and 50% alcohol, and with C.O.B. The results are shown in Appendix Table 8.

Appendix Table 8. *The time to end-point with* 68 *and* 50% *alcohol, and with* C.O.B. *of aseptically drawn single cow samples, of different alcohol instabilities, inoculated with a culture of* Str. lactis (*initial inoculum c.* 100,000* *per ml.*)

Cow no.	Giving a precipitate when fresh with an alcohol concentration of (%)	Time to end-point at 18° C. (hr.) Alcohol 68%	Alcohol 50%	C.O.B.	Final count per ml.* (×10⁶)
1	72	*a* 32	36	36	32
		b 32	36	36	16
2	76	*a* 28	30	30	130
		b 28	30	30	99
3	84	*a* 28	30	30	122
		b 28	30	30	162
4	84	*a* 36	38	40	230
		b 36	38	40	249
5	95	*a* 32	34	34	160
		b 32	34	34	180

a and *b* = duplicates.

* Colony count on yeast dextrose agar.

Though the results given in Appendix Tables 7 and 8 are too few to warrant firm conclusions, they indicate that the instability of fresh milk, to alcohol of high concentration, is a quality peculiar to the milk and is in no way related to its potential keeping quality as measured by C.O.B. or a lower concentration of alcohol.

The alcohol instability of fresh milk

It has been shown that keeping quality results using 50% alcohol to determine the end-point agree closely with those obtained with the C.O.B. test; even with 68% alcohol the relationship is still good, although occasional samples may, when fresh, precipitate with alcohol of this concentration. With alcohol concentrations in excess of 68% the relation to C.O.B. deteriorates and the proportion of milks unstable to alcohol when fresh, increases. Our data do not enable us to assert that the variations in the relationship to C.O.B. are solely due to an increase in the proportion of samples unstable to the higher concentrations of alcohol, but no other cause has appeared.

This instability of fresh milk to alcohol has already received considerable attention. Most of the investigations deal with samples from individual cows. Ayers & Johnson (4) have reviewed the earlier literature which indicates that precipitation with 68–70% alcohol of freshly drawn milk from individual cows is comparatively rare. More recently Mitamura (5), in a study of the behaviour towards alcohol of fresh milk from forty-two cows during a period of 12 months, found that of a total of 2704 tests 0·1, 0·6, 2·0, 4·0, 10·0% precipitated with 67, 69, 70, 71 and 80% alcohol respectively. Milk from fifteen individual cows was positive with 70% alcohol on the average for 12 days after calving and for 29 days before the end of the lactation, with a maximum of 28 days for the former and 42 days for the latter.

Benton & Alberry (6) recorded an instance where the fresh bulk milk from a herd of fifty-four cows gave a precipitate—presumably with 70% alcohol, though the exact concentration is not clear from the paper; the milk of forty-one of the fifty-four cows gave a positive reaction. The milk was apparently normal in every respect, and the reaction could not be ascribed to feeding, stage of lactation, nor to any pathological condition of the udders. According to Seekles & Smeets (7) precipitation of bulk fresh milk with 70% alcohol, a condition referred to as 'Utrecht abnormality', is not uncommon in Holland. Occasionally such fresh milk may also coagulate on boiling, but this is unusual, and for the majority of samples coagulation ultimately occurs following the proliferation of bacteria as with normal milk.

Mitamura (5) made a detailed study of the factors responsible for the alcohol instability of fresh, apparently normal, milk and reviewed the literature on the subject. He confirmed the findings of earlier workers (6, 8) that alcohol stability is mainly determined by the calcium-ion concentration. Increasing the calcium-ion concentration by the addition of calcium chloride induces alcohol instability, whereas decreasing the calcium ion by dialysis or by the addition of citrate, oxalate, phosphate or fluoride has the reverse effect. According to Mitamura (5) the total calcium oxide and magnesium oxide in alcohol-unstable milk, whether colostrum, end of lactation or mid-lactation milk, is approximately 16% higher than in normal milk; the sodium and phosphorus contents are constant throughout the lactation, but for a fixed weight of calcium, alcohol-stable milk has a higher phosphorus content than alcohol-unstable milk. Seekles & Smeets (7) also ascribe alcohol stability to the calcium-ion concentration, but found no significant difference between the total calcium ion or other mineral constituents of thirty 'Utrecht abnormal' and fifteen normal alcohol-stable milks.

The evidence regarding the effect of administration of calcium to cows is conflicting. Sommer & Binney (8) found that three cows fed 50 g. calcium carbonate daily gave milk which precipitated with 68% alcohol, though the calcium oxide content of the milk was unaffected. Mitamura (5), on the other hand, found that daily feeding of 150 g. bone meal (with or without the addition of cod-liver oil), or 50 g. calcium lactate, or 40 g. potassium phosphate (K_2HPO_4) had no effect on the alcohol stability of the milk. Seekles & Smeets (7) however, claim that the 'Utrecht abnormality' of milk from individual cows can be corrected by administration by mouth of 50 g. sodium citrate daily for several days or 30 g. in 10% sterile solution subcutaneously.

The evidence in the literature suggests that there is no definite relationship between the incidence of alcohol instability and feeding. According to Seekles & Smeets (7) the typical 'Utrecht abnormality' may be associated with too luxurious feeding, but this is not well established. Non-typical forms occur with milk from cows on a low plane of nutrition or when the diet consists mainly of poor hay, straw, or old silage. Weimar (9) induced alcohol instability by feeding mouldy silage and ascribed it to physiological disturbance leading to chemical changes in the milk. Mitamura (5) was also able to induce temporary alcohol instability by feeding a ration composed entirely of viscid fermented soya-bean cake; feeding protein-rich or protein-deficient rations for short periods, or sudden changes in coarse fodder were without effect on the alcohol stability of the milk.

Most of our studies were confined to observations of the incidence of bulk milk samples giving, in the absence of bacterial growth, a precipitate with 68% alcohol. Of the 618 samples examined in our experiments (Tables 1, 5; Appendix Tables 2, 7) only one gave

a precipitate with 68% alcohol at the time of transfer to 18 or 22° C. water-baths for keeping quality tests. Since these represent bulk samples from three widely separated areas in England and Wales, it may be assumed that the incidence of fresh bulk samples giving a precipitate with 68% alcohol is low, and that it is only in exceptional circumstances that the use of this concentration of alcohol will lead to anomalous results in keeping quality determinations. Further observations made at each of the three centres during January to March 1946, showed that with alcohol concentrations in excess of 68% the incidence of alcohol-unstable samples rises sharply. Moreover, there was a suggestion that instability towards these higher alcohol concentrations varied in the different areas; the highest incidence occurred in a limestone district, but no detailed study of the milk of individual cows composing the herds in this area was made.

It seems evident from the literature and from our own observations that bulk fresh milk which is in every other respect normal may occasionally give a precipitate with alcohol of a concentration as low as 68%. According to Seekles & Smeets (7) such abnormal milk, provided it is not associated with a pathological condition of the udder, is not objectionable from a nutritional or health aspect. A survey on a wide scale and at all seasons of the year, to establish the incidence of bulk fresh milk samples which give a precipitate with different alcohol concentrations, is desirable before the use of a concentration of alcohol in excess of 68% can be advocated with confidence as a measure of the end-point of keeping quality.

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418. DEVICE FOR PREPARING ROLL-TUBE CULTURES

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(With 1 Figure)

The roll-tube method for estimating bacterial counts was devised by Esmarch (1), but was soon afterwards superseded by the introduction of Petri plates which have several advantages for cultural and counting purposes. The roll-tube method, however, has been revived by several workers (2,3,4), and where a simple, rapid and economical method is required it is very useful, especially if low counts are being dealt with. For this reason it is particularly suitable for yeast and mould counts in butter and has been so used in this laboratory for many years. In addition to its regular use for this purpose, several series of comparisons have shown that the roll-tube method is capable of giving results which compare very well with Petri plate counts upon milk, using the usual dilution technique in both cases.

McKenzie & Lambert (5) stated that the time needed for hand rolling of the tubes is a marked deterrent to the use of the method. Some form of mechanical roller is essential and, as pointed out by Thomas, Jones & Lloyd (6), this must enable the rolling to be done quickly. Several workers have used an electrically driven six-tube rolling machine, first described by Mundinger & Woeckel (7), later used successfully by Damm (2), and then by Prouty, Bendixen & Swenson (4). Hirsch (8) recently described a similar apparatus made from Meccano parts. A simple form of roller has been used in this laboratory for several years, and is described in this paper.

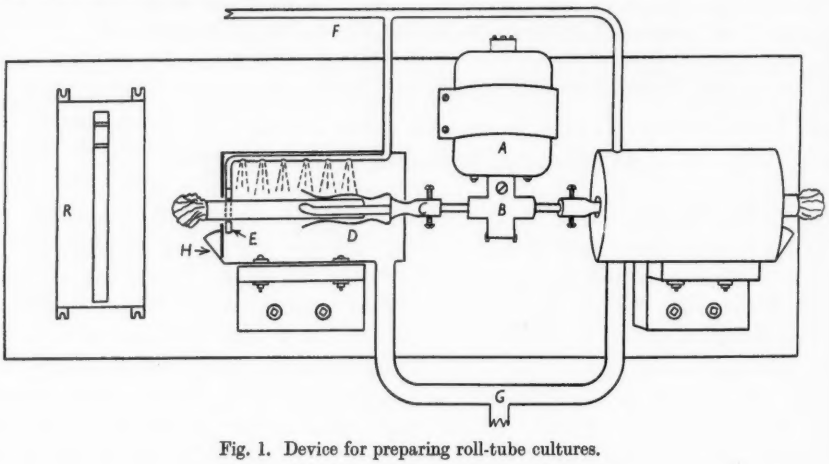

Fig. 1. Device for preparing roll-tube cultures.

The accompanying diagram (Fig. 1) shows a motor with fittings to roll two tubes, and one of the two rolling units appears in logitudinal section to illustrate details of construction. The device consists simply of a small electric motor *A* fitted with reduction gear *B* and rheostat *R* (not shown) to adjust the speed (Klaxon Geared Motor Unit, Frame

EK5UB3-W3). At each end of the shaft is attached* a brass coupling-sleeve *C* fitted with spring clips *D* to hold the test-tube while it is being rotated under a spray of water from the copper pipe *F*. Suitable clips may be made from spring brass or stainless steel, rather larger than burette clips (as listed by Griffin and Tatlock, Cat. No. 19-870). Each of the four prongs is about 2 in. long, and if there is any tendency for the test-tube to slip out, a short piece of rubber tubing slipped over one prong gives a firmer grip. In the diagram, cylindrical water-tight covers of brass or copper are shown, with detachable rubber unions to water pipes, inlet *F* and outlet *G*. These connexions enable the device to be mounted on the wall close to where culturing operations are carried out. By fitting small internal brackets with screws, the end of the cylinder farthest from the motor is detachable, and is provided with a cone-shaped piece of metal *H* to collect any splash of water and return it by a small hole to the cylinder. A simpler alternative is to mount the roller over a sink and provide a suitable metal shield. A ring *E* may then need to be fixed to the end of the inlet water tube to support the plugged end of the test-tube.

Compared with the Mundinger machine this apparatus is simpler but of at least equal capacity, because by cooling the tubes with water the agar solidifies very quickly and the tubes can be removed from the roller after about 15–20 sec. Another advantage is that ordinary $6 \times \frac{3}{4}$ in. test-tubes may be used instead of the special constricted tubes required in the Mundinger machine. After rolling, the tubes are left in a horizontal position for some hours (overnight) to ensure the agar setting firmly. In a previous paper from this laboratory (3), a medium with agar of 3% strength was recommended to ensure good setting properties in the tubes. The strength needs to be adjusted to suit the quality of agar in use, but a final concentration of about 2% after inoculation is satisfactory.

The counting of roll-tube cultures is facilitated by good lighting over a dark background. A grease pencil line may be drawn along the tube to indicate a starting point. If necessary the area to be counted may be quartered by slipping the test-tube into a close-fitting copper tube from which one-quarter of the circumference has been removed. Because of their size and opacity no difficulty is experienced in counting yeast colonies, but for small colonies in milk cultures a 4 in. magnifying lens is essential to obtain accuracy.

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* If some flexibility is desired a short piece of stout rubber tubing may be used to connect the shaft to the sleeve *C*.

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637.127.6

419. THE PHOSPHATASE TEST OF ASCHAFFENBURG AND MULLEN

USE OF PERMANENT COLOUR STANDARDS AND COMPARISON WITH THE KAY-GRAHAM TEST

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(With 3 Figures)

Aschaffenburg & Mullen (1) have recently developed a phosphatase test based on the substrate *p*-nitrophenylphosphate. On hydrolysis of this substrate by milk phosphatase, free *p*-nitrophenol is liberated which, being yellow in alkaline solution, serves as a direct indicator of the extent of enzyme activity. Aschaffenburg & Mullen match the colours of test samples against boiled milk standards containing known quantities of *p*-nitrophenol. These standards must be prepared afresh for each batch of tests, but we have found that the need for their frequent preparation can be eliminated by the use of a simpler and more accurate technique of colour assessment, consisting of a comparison of the test colours in reflected light with those of suitable yellow glass standards. This technique has been used for a direct comparison between the test of Aschaffenburg & Mullen and the official one of Kay-Graham (2).

EXPERIMENTAL

Technique

The technique used for the new test was that given by Aschaffenburg & Mullen, except that the tubes containing the test samples were matched against the yellow glasses of the Lovibond Comparator Disks KDA and KDAB,* normally used in the estimation of chlorine in water by the *o*-tolidine method for which they are calibrated in p.p.m. chlorine. The colour standards used were 0·1, 0·2, 0·3, 0·4, 0·5 (disk KDA) and 0·15, 0·25 p.p.m. chlorine (disk KDAB). The comparisons were made in a Lovibond Comparator of the simple type used in the resazurin test. The yellow colours of the glasses gave an excellent match with the colours developed by the liberation of *p*-nitrophenol. Matching was found at least as easy as that of the blue colours in the Kay-Graham test. The tubes used were those conforming to B.S.S. No. 625 (1935) 152/16, having an internal diameter of 13·5 ± 0·5 mm. and being accurately marked at 10 ml., with rubber stoppers to fit. They should be made of colourless glass.

The contents of the tubes should always be well mixed immediately before a reading is taken. A tube containing 2 ml. of boiled milk and 10 ml. of buffer substrate solution served as a blank; this corresponds to the standard 0 of Aschaffenburg & Mullen. Test samples of properly pasteurized milk tended to show a slight greyish tinge when compared with this cold unincubated standard. Towards the end of this work, it was found that better matching could be obtained by incubating the boiled-milk standard with the test samples.

* Made by The Tintometer Ltd., Salisbury.

In comparing the test colours with those of the standard glasses, readings falling between two standards were estimated and recorded by affixing a plus or minus sign to the figure for the nearest standard. The same procedure was used in the Kay-Graham test, using the nine-colour standard disk A.P.P. 9 in the Lovibond 'All purposes' comparator with cell of 25 mm. depth. The substrate used was provided by Dr Aschaffenburg and gave no appreciable colour when made up with the buffer. We found that the buffer substrate solution did not deteriorate when kept for 3 days in a dark stoppered bottle in the refrigerator at a temperature of approx. 0° C.

Milk samples

182 samples of milk were subjected to the Kay-Graham test and to the test of Aschaffenburg & Mullen, in which readings were taken after incubation for 30 min. and 2 hr. The samples were made up of ninety-three plant-pasteurized milks from several creameries and of eighty-nine samples prepared in the laboratory which consisted largely of underheated milks and of properly pasteurized samples containing small quantities of raw milk, so as to spread the results over the critical range.

RESULTS

The 30 min. test

Table 1 shows the relationship between the results of the test of Aschaffenburg & Mullen after incubation for 30 min. and those of the Kay-Graham test.

Table 1. *Comparison between the Aschaffenburg & Mullen test and the official Kay-Graham test. 30 min. incubation*

A. & M. test p.p.m. chlorine (estimated values)	No. of samples	Kay-Graham test (Lovibond Blue Units) 1·5 – to 1·5 +	1·8 – to 1·8 +	2·1 – to 2·1 +	2·3	2·5 – to 2·5 +	3·0 – to 3·0 +	4·0 – to 5·0	5·0 + to 6·0 +
No colour, or trace only	90	56	34	—	—	—	—	—	—
0·1 –	28	7	10	7	2	—	2	—	—
0·1	21	—	—	4	1	7	9	—	—
0·1 +	13	—	—	—	—	—	13	—	—
0·15 – to 0·15 +	14	—	—	—	—	—	7	6	1
0·2 – to 0·2 +	10	—	—	—	—	—	1	6	3
0·25 and above	6	—	—	—	—	—	—	1	5
Totals	182	63	44	11	3	7	32	13	9

Taking the accepted figure of 2·3 L.B.U. in the Kay-Graham test as the limit for proper pasteurization, the results indicate that with the test of Aschaffenburg & Mullen:

(1) Milks giving readings equal to or in excess of 0·1 p.p.m. chlorine are improperly pasteurized.

(2) Milks giving no colour or only a trace are properly pasteurized.

(3) Milks giving intermediate readings must be regarded as doubtful. They should be reincubated for a further 90 min., and then examined by the more searching 2 hr. test.

The 2 hr. test

When the incubation time in the test of Aschaffenburg & Mullen was extended to 2 hr. and the results were compared with those of the Kay-Graham test, a more precise classification of the samples was possible as shown in Table 2.

Table 2. *Comparison between the Aschaffenburg & Mullen test and the official Kay-Graham test. 2 hr. incubation*

A. & M. test p.p.m. chlorine (estimated values)	No. of samples	Kay-Graham test (Lovibond Blue Units) 1·5 – to 1·5 +	1·8 – to 1·8 +	2·1 – to 2·1 +	2·3	2·5 – to 2·5 +	3·0 – to 3·0 +	4·0 – to 5·0	5·0 + to 6·0 +
No colour, or trace only	85	56	29	—	—	—	—	—	—
0·1 – to 0·1	23	7	12	3	1	—	—	—	—
0·1 +	4	—	2	2	—	—	—	—	—
0·15 – to 0·15	11	—	1	6	2	2	—	—	—
0·15 + to 0·20 +	8	—	—	—	—	4	4	—	—
0·25 – to 0·25 +	25	—	—	—	—	1	23	1	—
0·30 – to 0·30 +	15	—	—	—	—	—	5	8	2
0·40 – to 0·40 +	10	—	—	—	—	—	—	4	6
0·55	1	—	—	—	—	—	—	—	1
Totals	182	63	44	11	3	7	32	13	9

The table shows that a value of 0·15 p.p.m. chlorine corresponds to the limiting value of 2·3 L.B.U. of the Kay-Graham test. If this value is taken as the limit for satisfactory pasteurization, only two samples out of 182 would have satisfied the test of Aschaffenburg & Mullen which failed to pass the Kay-Graham test, and these were borderline cases with readings of 2·5 L.B.U. Also, on this basis, no sample was failed, which would have satisfied the official Kay-Graham test.

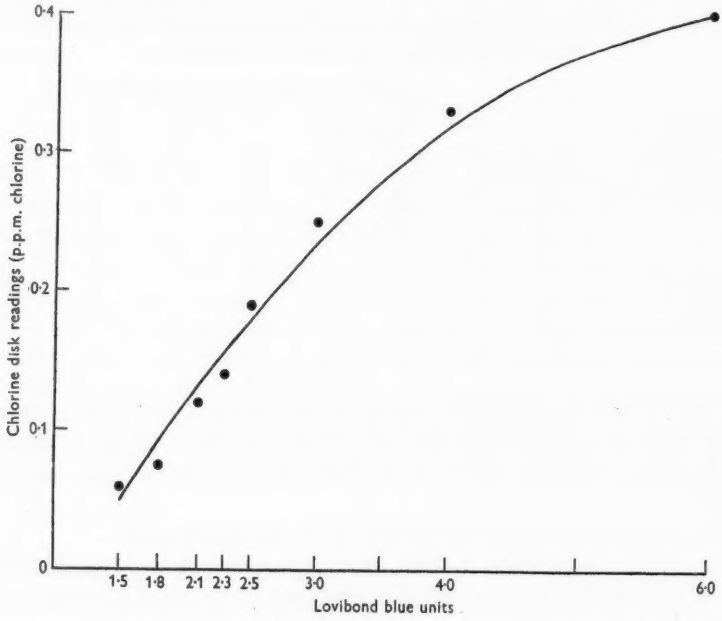

Fig. 1. Relationship between the Aschaffenburg & Mullen (2 hr.) test and the Kay-Graham test.

The results are summarized in Fig. 1 in which are plotted the mean values for the Aschaffenburg & Mullen readings for each range of Lovibond Blue Units. The value of 0·04 p.p.m. chlorine was assigned to the reading 'No colour or trace only'.

Comparison between the chlorine disk readings and the Aschaffenburg & Mullen standards

The aim of this work was to compare the Aschaffenburg & Mullen test, using permanent colour standards, with the official Kay-Graham test, and, in particular, to establish a limiting value for the former test equivalent to 2·3 L.B.U. All the samples were therefore regarded as unknowns, though the history of many was in fact known.

It can be seen from Table 1 (30 min. incubation) that, of ninety-two samples giving higher readings than 'No colour, or trace only', thus exceeding the limit proposed by Aschaffenburg & Mullen, thirty-one satisfied the Kay-Graham test. Table 2 (2 hr. incubation) shows that, of seventy-four samples giving readings above the Aschaffenburg & Mullen standard 'I', which we assume to be equivalent to 0·1 p.p.m. of chlorine, thirteen satisfied the Kay-Graham test.

While it would appear from the above that the standards proposed by Aschaffenburg & Mullen are unduly stringent, it should be stated that the five samples which, in the 30 min. test, gave readings of 0·1 p.p.m. with corresponding L.B.U. readings of 2·1 – to 2·3 were in fact known to have been underheated. Also, a large proportion of the samples giving readings of more than 'No colour, or trace only' but less than 0·1 p.p.m. were known to have been slightly underheated. Similarly, the thirteen samples which failed on the standards set by Aschaffenburg & Mullen for the 2 hr. incubation period and satisfied the Kay-Graham test were known to have been slightly underheated. Of these milks, 10 gave L.B.U. readings of 2·1 – to 2·3; they thus satisfied the official regulations, but the readings were higher than those normally encountered in commercial practice. However, some allowance should clearly be made for methods in which there exists the possibility of different visual evaluation of colour readings.

Calibration of the disks in terms of p-*nitrophenol*

The chlorine disks were calibrated in terms of μg. of *p*-nitrophenol/ml. of milk by comparison with boiled-milk standards containing known quantities of *p*-nitrophenol, as shown in Fig. 2. These values are mean readings by two or more workers. The standards were made up several times, using different boiled milks, and the comparisons with the permanent colour standards were made in north daylight immediately after preparation of the standards. It was noted in the course of this work that the colours of the *p*-nitrophenol standards increased in intensity on standing at room temperature, whether protected from light or not; this was quite marked after 90 min.

Fig. 3, which is derived from Figs. 1 and 2, shows the relationship between the colour readings of Aschaffenburg & Mullen's 2 hr. test in terms of *p*-nitrophenol and those of the Kay-Graham test. This relationship appears to be a linear one. Though we have not actually measured all the control values of the Kay-Graham tests, it can be reasonably assumed that these will be about 1·2 L.B.U. on the average. When the straight line is drawn through this point on the abscissa, the following equation is derived:

$$\mu\text{g. } p\text{-nitrophenol/ml. milk} = (\text{L.B.U.} - 1{\cdot}2) \times 9{\cdot}5.$$

Special disk for the phosphatase test of Aschaffenburg & Mullen

Whilst the closest possible colour matching was desirable in the experiments reported above, it was felt that, for routine purposes, a disk containing fewer colour standards would suffice.

Such a disk* was kindly prepared by Messrs Tintometer Ltd., to whom we wish to express

* Lovibond disk APTW.

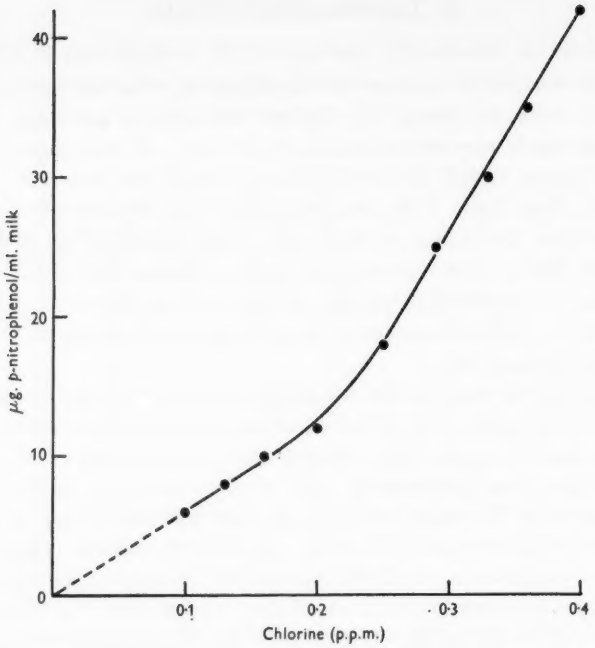

Fig. 2. Calibration of chlorine disk in terms of μg. *p*-nitrophenol/ml. milk.

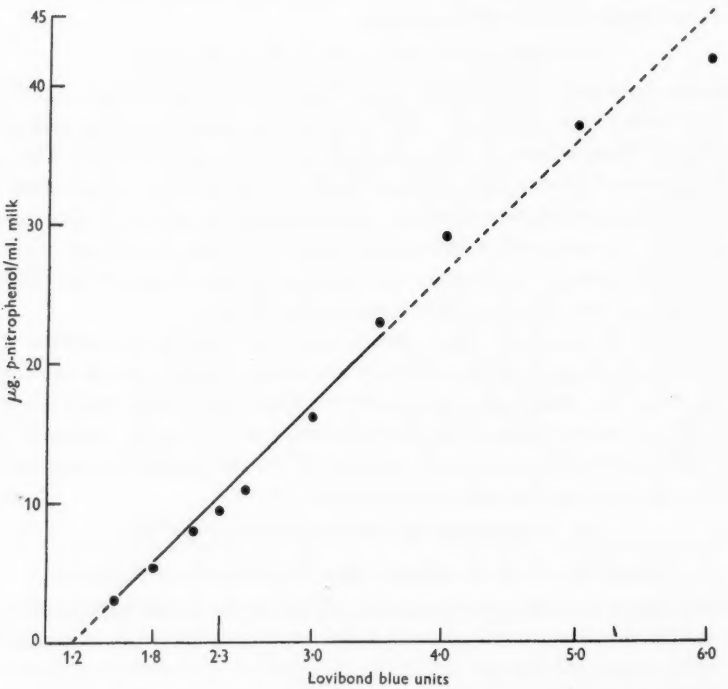

Fig. 3. Relationship between the μg. *p*-nitrophenol/ml. milk of the Aschaffenburg & Mullen (2 hr.) test and the Lovibond Blue Units of the Kay-Graham test.

our gratitude. It is calibrated in terms of μg. *p*-nitrophenol/ml. of milk as shown in Table 3, which also gives the corresponding chlorine values and the mean Kay-Graham test readings observed in this laboratory to correspond to the readings of the 2 hr. test.

Table 3

μg. *p*-nitrophenol per ml. of milk	Chlorine (p.p.m.)	L.B.U.
6	0·1	1·8+
10	0·15	2·3
18	0·25	3·2
42	0·40	6·0

The suggested scheme of interpretation of results based on the use of the new disk is shown in Table 4.

Table 4

30 min. incubation	μg. *p*-nitrophenol per ml. of milk	2 hr. incubation
Properly pasteurized	0 or trace	Properly pasteurized
Doubtful	6	
Under-pasteurized	10	
	18	Slightly under-pasteurized
	42	Under-pasteurized
	Over 42	Grossly under-pasteurized

The more searching 2 hr. test should be applied whenever time permits, but examination of the tubes after 30 min. is useful in that it will reveal any major fault in processing and permits, in cases of doubt, correct classification by the simple expedient of re-examination after incubation for a further 90 min.

The technique of the Aschaffenburg & Mullen test is much simpler than that of the Kay-Graham test; fewer operations are required, and the test is much less liable to be affected by interfering substances than the official test. This, coupled with the fact that results are obtained in 2 hr. and possibly in 30 min., makes the Aschaffenburg & Mullen test admirably suited for routine laboratory purposes. It is suggested that this test should be further examined as a possible alternative to the official test.

SUMMARY

1. A method has been developed for measuring the intensity of colour produced in the Aschaffenburg & Mullen phosphatase test in which use is made of permanent colour standards.

2. Using this method, the relationship between this phosphatase technique and the official Kay-Graham test has been investigated.

3. The limiting standards corresponding to 2·3 Lovibond Blue Units in the Kay-Graham test have been established for the 30 min. and 2 hr. procedures of Aschaffenburg & Mullen.

4. The advantages of the improved technique of the Aschaffenburg & Mullen test are discussed.

The authors wish to thank Dr Aschaffenburg for his helpful interest and provision of the buffer substrate, and the Directors of C.W.S. Limited for permission to publish this work.

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637.127.4

420. THE COLLOIDAL PHOSPHATE OF MILK

I. COMPOSITION AND TITRIMETRIC ESTIMATION

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The colloidal phosphate of milk has been variously identified as dicalcic (1), as a mixture of dicalcic and tricalcic (2), and as tricalcic or predominantly so (3,4). The correctness of the last identification, though apparently confirmed by many workers during the last fifteen years (5, 6, 7, 8), has now been challenged. In a recent critical review of the evidence to date (9), it is objected that (*a*) fixity of composition of the colloidal phosphate is irreconcilable with the degree of variation of the insoluble inorganic Ca/P ratio calculated from recorded data, especially those of Van Slyke & Bosworth, on the composition of milk and milk sera, and (*b*) that the oxalate titration of milk, etc. (5), hitherto held to determine tricalcium phosphate only, may, in reality, measure total colloidal phosphate. The first contention is hardly conclusive. No great constancy can be expected in a Ca/P ratio based on analytical differences between milk and milk ultrafiltrate—never a satisfactory serum—and on an assumed constancy of composition of calcium caseinate. Significantly, the average value of the ratio corresponds to tricalcium phosphate. The second objection, challenging as it does the reliability of the generally accepted oxalate method of estimating colloidal tricalcium phosphate, is more serious. If, as is now suggested, conversion of colloidal dicalcium phosphate to tricalcium phosphate can occur during the titration of unoxalated milk with alkali, then the tricalcic content of the colloid thus determined is certainly liable to over-estimation.

The validity of this contention could best be tested, it seemed, by a comparison of the tricalcium phosphate values given by the Ling oxalate titration with those furnished by some satisfactory alternative method, free from the criticized treatment of the colloid with alkali. The present paper describes a new form of oxalate titration which fulfils this condition, and it records and discusses a series of comparative determinations obtained by its aid. Only the apparent chemical composition of the colloidal phosphate will be dealt with here, the further and more complex question of the nature of the association of colloidal phosphate with caseinate being reserved for later treatment.

METHOD OF INVESTIGATION

When a soluble oxalate, say potassium oxalate, is added to milk, an alkalinity is developed, mainly by conversion of colloidal di- and tricalcium phosphate into the corresponding potassium salts, and, to a much lesser degree, by comparable changes in the soluble salts and by the conversion of calcium caseinate into the more highly dissociated potassium compound. The total alkalinity thus developed can be determined by back-titration with acid to the original pH of the milk. By correction of this figure for the contribution made by the soluble constituents of the milk and for that due to the caseinate change (determinable by similar titrations of oxalated whey and of calcium caseinate solutions respectively), the net alkalinity arising from the action of oxalate on the colloidal phosphates can be

obtained. The relative amounts of primary and secondary phosphate in the final mixture which results from the back-titration with acid to the original pH of the milk can be read off from Sorensen's table of phosphate buffers(10); the total amount of phosphate in colloidal form can be obtained from the difference between the inorganic phosphate contents of milk and of milk dialysate; whence the composition of the colloidal phosphate originally present in the milk can be calculated as follows:

Example

pH of milk 6·66

% casein 2·80

$$\frac{[KH_2PO_4]}{[K_2HPO_4]+[KH_2PO_4]} = 0{\cdot}59 \text{ at pH } 6{\cdot}66.$$

Total colloidal phosphate 0·95 mM. H_3PO_4/100 ml.

Back-titration of oxalated milk 1·54 ml. N/1 acid/100 ml.

Back-titration of oxalated whey 0·07 ml. N/1 acid/100 ml.

Back-titration of oxalated 2·8% Ca caseinate 0·15 ml. N/1 acid/100 ml.

If D and T represent the concentrations of dicalcium and tricalcium phosphate respectively, expressed as millimols H_3PO_4/100 ml. milk:

$$D + \quad T = 0{\cdot}95,$$

$$0{\cdot}59\,D + 1{\cdot}59\,T = 1{\cdot}54 - 0{\cdot}07 - 0{\cdot}15 = 1{\cdot}32,$$

so that $D = 0{\cdot}19$, $T = 0{\cdot}76$, or 80% of the calcium phosphate is tricalcic.

The composition of the colloidal phosphate furnished by the foregoing—or indeed any similar—method is based on the assumption that calcium caseinate possesses, at the pH of milk, the same calcium content whether in association with colloidal phosphate or not. Without this assumption, the problem is indeterminate.

EXPERIMENTAL

In applying this method, the following precautions were taken. Back-titrations of the oxalated samples were made, under rapid mechanical stirring, with a N/20-acetic acid N/10-sodium acetate buffer mixture of pH 5·0 (approx.) to obviate the risk of local casein coagulation. This acid buffer was standardized against N/20-K_2HPO_4 solution by titration to the various pH end-points (6·5–7·1) likely to be encountered in normal milk samples. The back-titrations found for each milk, whey, etc., were expressed in terms of the equivalent phosphate by use of the appropriate conversion factor. The applicability of the Sorensen pH values to phosphate mixtures in oxalated milk was confirmed by pH measurements on known phosphate-oxalate-caseinate mixtures of comparable composition. All pH measurements were made by the glass electrode.

Titrations were carried out on 5 ml. samples of milk and whey. Addition of 0·2 ml. neutral saturated potassium oxalate solution was found to increase the pH of milk by about 1 unit (0·8–1·5), and that of whey by about 0·05. The corresponding back-titrations averaged about 1·60 and 0·08 ml. N/1 acid/100 ml. respectively.

Caseinate blanks were determined on 3% calcium caseinate solutions (Hammarsten casein) of pH 6·65, containing 3 mM./l. $CaCl_2$ and 70 mM./l. NaCl and thus, in ionic content, corresponding approximately to milk. Addition of the usual proportion of oxalate raised the pH of this solution by 0·25, and subsequent addition of acid at the rate of 0·055 ml.

N/1 acid/g. casein was required to restore the pH to its original value. (This figure is somewhat lower than that—viz. 0·08 ml. N/1 acid/g. casein—calculated from Sandelin's results (11) for the base-binding capacities of casein for sodium and calcium hydroxides at pH 7·0. Probably the discrepancy is mainly due to the diminished dissociation of potassium caseinate in the presence of relatively high salt concentrations of the order of those encountered in oxalated milk.) The caseinate correction for any particular milk was obtained by multiplying its casein percentage (taken as 78·5% of the protein determined by formaldehyde titration) by 0·055.

As both the whey and caseinate titrations are relatively small, no significant loss of accuracy was thought likely to result from the use of the more convenient rennet whey instead of milk dialysate for the former, or from the use of the formaldehyde titration to estimate the caseinate content of milk.

Total colloidal phosphate was taken as the difference between the total and the soluble inorganic phosphate of milk, the former being determined in the trichloracetic acid filtrate of milk, and the latter in milk dialysate (in certain cases also in rennet whey), using the colorimetric methods described elsewhere (12). Milk dialysate was prepared by 48 hr. dialysis of milk at 0–5° C. against 1/50 volume of water, and rennet whey was prepared as described by Ling (5). (The phosphate content of whey is somewhat lower than that of milk dialysate (12,13); the difference averages about 6% in the present series of experiments. As a result, the colloidal phosphate content of milk is liable to be correspondingly overestimated—by about 7%—if whey is used instead of milk dialysate for the determination of the soluble phosphate of milk.)

The Ling titration was carried out as described by Ling (5). Modifications introduced in the course of the present work are dealt with as they arise.

To ensure as great a variety of types and contents of colloidal phosphate as possible, milks of individual cows were taken for examination, mostly fresh morning milks of animals from the College Dairy Shorthorn herd. Forty samples were examined, one-half during May–June 1949, the other half during September–October 1949. Before examination, fat was removed by separator from all samples to facilitate the preparation of sera and to minimize the volume corrections associated therewith. All sera analysed were corrected for the volume of protein removed in their preparation from milk (assuming protein density 1·6) and for any incidental dilution.

RESULTS

Table 1 gives the results obtained for twenty milk samples, phosphate contents and titrations being recorded as millimols H_3PO_4/100 ml., and the new oxalate titrations corrected for the whey and caseinate contributions. The tricalcium phosphate content of the inorganic colloid determined by the new method averages 89%. Individual departures from the average are, with one exception, not very great in view of the number of estimations involved. Most, but not all, of the colloidal phosphate appears thus to be tricalcic.

The average percentage of tricalcium phosphate yielded by the Ling titration (100%) is decidedly higher than this figure. It agrees very closely with the figure (93%) calculated from Ling's data on mixed milk (5) when account is taken of the fact that Ling's estimates of total colloidal phosphate are about 7% too high, because of the use of whey instead of milk dialysate for the determination of soluble phosphate.

Table 1

Sample	pH	% casein	mM. H_3PO_4/100 ml. Total colloidal inorganic phosphate	Ling titration	New oxalate titration	% colloidal phosphate in form of $Ca_3P_2O_8$ calculated from Ling titration	New oxalate titration
1	6·68	3·02	0·67	0·825	1·01	123	90
2	6·71	3·82	0·71	0·83	1·02	117	86
3	7·04	2·55	0·76	0·77	1·01	99	95
4	6·82	2·96	0·78	0·845	1·20	108	102
5	6·71	2·77	0·845	0·965	1·45	114	114
6	6·71	2·92	0·845	0·81	1·34	96	100
7	6·68	2·83	0·86	0·755	1·19	88	78
8	6·77	2·56	0·865	0·84	1·23	97	88
9	6·65	2·89	0·865	0·98	1·34	113	94
10	6·66	2·89	0·87	0·955	1·39	110	99
11	6·69	2·67	0·88	0·80	1·30	91	89
12	6·76	3·37	0·885	0·82	1·32	93	94
13	6·70	3·24	0·90	1·15	1·28	78	85
14	6·80	3·40	0·91	1·085	1·18	119	77
15	6·81	2·77	0·97	0·89	1·27	92	79
16	6·78	2·74	0·97	0·87	1·35	90	85
17	6·67	2·40	0·98	0·875	1·39	89	82
18	6·69	2·74	1·02	0·915	1·375	90	76
19	6·81	2·72	1·14	1·01	1·51	89	81
20	7·04	3·20	1·275	1·18	1·56	93	84
					Average	100	89

The Ling titration

The high results obtained by the Ling titration appeared at first sight to lend some support to ter Horst's criticism of this method. Conversion of colloidal dicalcium phosphate to tricalcic during milk titration could certainly account for such high results. A simpler explanation was, however, available. It has long been known that the pH of milk, neutralized to phenolphthalein, is considerably higher than that of a protein-free salt mixture of similar composition brought to the same phenolphthalein tint (14). For milk and whey, under the conditions of the Ling titration, the corresponding differences in the pH of the end-points arising from this 'protein error' are found to be about 0·5 pH. Conversion of soluble phosphate to tricalcic will, therefore, be more complete, and the so-called 'overrun' accordingly greater, in the milk titration than in the whey titration. The Ling method for the estimation of the colloidal tricalcium phosphate present in the milk, which involves both the difference between these titrations and the assumption that the 'overrun' is the same in each, will thus yield results which are increased by the difference in the two 'overruns'. As will be seen later (Table 2), the error arising from this source is far from negligible. It amounts, on the average, to about 10% of the Ling titration, or sufficient to account for almost the entire difference between the results obtained by the Ling and by the new oxalate titrations. As already pointed out (4) in connexion with a procedure essentially similar to Ling's, an additional small correction for the alkalinity developed on oxalation of a calcium caseinate solution previously neutralized to phenolphthalein, would still further improve the results. It appears unnecessary, then, to assume, as postulated by ter Horst, any appreciable conversion of colloidal dicalcium phosphate (of which relatively little seems to exist in milk) into tricalcium phosphate during milk titration.

Modified Ling titration

Table 2 gives the results for ten more milk samples of comparative estimations of tricalcium phosphate by the new oxalate procedure, by the usual Ling titration, and by a modification of the latter in which both milk and whey are titrated to the same end-point at approximately pH 8·65. This last was conveniently accomplished by the addition of 12 times as much phenolphthalein to the milk as to the whey, and titration of both to the same appropriate standard tint. Following a suggestion of Thomé(15) to improve the accuracy, an interval of 2 min. was allowed with unoxalated samples between the first attainment of the standard tint and the completion of the titration.

Table 2

			As mM. H_3PO_4/100 ml.				% colloidal phosphate in form of $Ca_3P_2O_8$ calculated from		
Sample	pH	% casein	Total colloidal inorganic phosphate	Ling titration	Modified Ling titration	New oxalate titration	Ling titration	Modified Ling titration	New oxalate titration
1	6·69	3·84	0·74	0·82	0·73	1·195	111	99	92
2	6·68	3·26	0·83	0·97	0·85	1·29	117	102	95
3	6·85	2·57	0·99	0·98	0·85	1·32	99	86	83
4	6·59	3·12	0·99	1·06	0·98	1·62	107	99	99
5	6·69	2·87	1·13	1·09	0·93	1·505	96	82	74
6	6·69	2·87	0·99	0·94	0·91	1·47	95	92	89
7	6·65	3·01	1·08	1·045	0·98	1·615	97	91	88
8	6·66	3·21	1·04	1·14	1·05	1·59	110	101	91
9	6·64	2·99	1·32	1·245	1·17	1·94	94	89	84
10	6·71	3·65	1·40	1·345	1·23	2·05	96	88	88
						Averages	102	93	88

As before, the results obtained by the new oxalate procedure indicate that most of the colloidal phosphate (here 88%) corresponds to tricalcic, an estimate resonably well confirmed by the modified Ling titrations (average 93% tricalcic). The original Ling procedure once more yields distinctly higher values (102%).

A final group of ten samples (results not tabulated) examined by the new oxalate titration alone gave 86% (75–105) as the average percentage of the colloidal phosphate present in the tricalcic form. For all forty individual milks the average, thus determined, is 88%.

DISCUSSION

The figure (88%) obtained for the average percentage of tricalcium phosphate in the colloidal phosphate by the new oxalate titration confirms the writers' early view that most, but probably not all, of the colloidal phosphate occurred in this form. The range of individual percentages of tricalcium phosphate found (viz. 75–105%, with one exception, corresponding to $[Ca]/[PO_4]$ ratios 1·38–1·57) is sufficiently narrow, given the very varied colloidal phosphate contents of the milks examined and the moderate accuracy of the method used, to suggest a fairly constant composition of the colloidal phosphate, and to tell against the recent hypothesis(16) of a colloidal phosphate of indefinite composition resulting from independent adsorption of its basic and acidic groups.

The somewhat higher estimates of the tricalcium phosphate content of the colloid arrived at through the Ling titration seem to arise chiefly from the divergent pH value of the milk and whey end-points employed. Where precautions are taken to bring these into

conformity, agreement between the results obtained by the Ling titration thus modified and the new oxalate method are reasonably good.

As to the nature of the remaining 12% of the colloidal phosphate, the obvious alternatives are magnesium phosphate, dicalcium phosphate and a mixture of the two. Magnesium phosphate has been suggested as a possible constituent of the colloid (13, 5), but the relatively low proportion of magnesium found in the centrifugally separated caseinate-phosphate complex of milk (6), and the feeble complex building properties of magnesium phosphate with caseinates (unpublished observation) both tell against its presence in any appreciable amount.

It is clear, on the other hand, from the presence of insoluble dicalcium phosphate as the major inorganic constituent of separator slime (17), that this substance is present (to some degree at least) in milk. Secondary alkaline earth phosphates as such appear, moreover, to be as capable as tertiary phosphates of forming complexes with caseinates. The phosphatic constituent of the barium caseinate-barium phosphate complex has been found, for example, to be mainly dibarium phosphate (unpublished result). The probabilities thus appear to favour dicalcium phosphate as the chief minor constituent of the colloidal phosphate of milk.

SUMMARY

1. A new type of oxalate titration permitting determination of the nature of the colloidal phosphate of milk has been developed.

2. About 88% of the colloidal phosphate appears to be tricalcic, the remainder is thought to be dicalcic.

3. Estimates of tricalcium phosphate by the Ling titration are distinctly higher than the foregoing. This would seem to be due mainly to the divergent pH values to which milk and whey are titrated in this method. There is no evidence of any conversion of colloidal dicalcium to tricalcium phosphate during the Ling titration, as had been suggested.

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421. HAEMOLYTIC STREPTOCOCCI IN CHEESE

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INTRODUCTION

In a paper by Mattick & Shattock (1) it is stated that: 'The suspicion exists in the minds of those who investigated cases of gastro-enteritis that streptococci of the faecal type isolated from suspected cheese may have been responsible for the disease.' They examined thirty cheeses and found that the faecal types of streptococci were common, and that the haemolytic varieties were rare. Since then the question has arisen as to the identity of the haemolytic streptococci found in cheese examined for the presence of the pathogenic streptococci. While we have never had occasion to examine cheese suspected of harbouring pathogenic organisms, the presence of haemolytic varieties of streptococci, their identity, and their role in cheese œcology is of general interest.

MATERIALS AND METHODS

Isolation of haemolytic streptococci

Four varieties of cheese were tested, two Roqueforts from France, five Danish Blues from Denmark, four Stiltons and five Cheddars from England. Approximately 1 g. taken from the centre of a slice of cheese was ground with aseptic precautions in a mortar containing a small quantity of silver sand and 10 ml. of Ringer's solution. Serial dilutions of the emulsified cheese were made in Ringer's solution and plated out in 5% horse-blood nutrient agar. The plates were incubated at 37° C. for 24 hr., the number of haemolytic colonies counted, and a few colonies picked off and purified by streaking on blood agar.

Physiological tests

Growth at 45° C.*; survival at* 60° C. *for* 30 *min.* Shattock (2).
Growth at pH 9·6. Shattock & Hirsch (3).
Arginine hydrolysis; growth in 6·5% *sodium chloride broth.* Abd-el-Malek & Gibson (4).
Carbohydrate fermentation. Peptone water with 0·5% (w/v) of the carbohydrate.

Serological tests

Precipitin ring tests were made on HCl extracts of all strains using serum prepared against the various Lancefield groups of streptococci. Extracts which failed to react were concentrated with ethanol (Mattick & Shattock (1)) and retested.

RESULTS

Of the sixteen cheeses examined eight contained haemolytic streptococci, the number present varying from 1×10^3 to $1{\cdot}7 \times 10^6$/g. Haemolytic streptococci were isolated from all four varieties of cheese sampled (Table 1).

Table 1. *Distribution of haemolytic streptococci among four types of cheese*

Type of cheese	No. examined	No. containing haemolytic streptococci
Danish Blue	5	3
Stilton	4	1
Roquefort	2	1
Cheddar (English)	5	3
Totals	16	8

Table 2. *Physiological characteristics of haemolytic streptococci isolated from cheese*

	Division of strains					
	1	2	3	4	5	6
Catalase test	−	−	−	−	−	−
Arginine hydrolysis	+	+	+	+	+	+
Growth in 6·5% salt	+	+	+	+	+	+
Survival at 60° C. for 30 min.	+	+	+	+	+	+
Haemolysis	+	+	+	+	+	+
Gelatin liquefied	+	−	−	−	−	−
Complete reduction of litmus milk 24 hr.	+	+	−	−	−	−
Growth at pH 9·6	+	+	+	+	−	−
Growth at 45° C.	+	+	+	+	+	+
Carbohydrate fermentation:						
Mannitol	+	+	+	+	−	−
Sucrose	+	−	+	+	−	−
Raffinose	−	−	−	−	−	−
Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+
Arabinose	−	−	−	−	+	−
Xylose	+	−	−	−	−	−
Sorbitol	+	+	+	−	−	−
Glycerol	+	+	+	+	+	+
Salicin	+	+	+	+	+	+
Inulin	−	−	−	−	−	−
Aesculin	+	+	+	+	+	+
Starch	−	−	−	−	−	−
No. of strains in each division	14	2	3	3	7	9
Source of strains	Danish Blue		Stilton		Cheddar	Roquefort
No. of cheeses	3		1		3	1
Designation of strains	*Str. faecalis* var. *zymogenes*				*Str. durans*	

Identity of haemolytic streptococci

Serological. Extracts of all the strains of haemolytic streptococci isolated from cheese reacted with group D serum but not with serum prepared against the other Lancefield groups. The only aberrant strains requiring concentration with ethanol were those isolated from the Roquefort cheese.

Physiological. The results of the tests on thirty-eight strains of haemolytic streptococci are given in Table 2. The Sherman tests, growth in broth containing 6·5% sodium chloride, survival at 60° C. for 30 min., growth at pH 9·6 and at 45° C., indicate that the organisms belong to the enterococci. Haemolysis, liquefaction of gelatin and fermentation of mannitol, sucrose, raffinose, divide these streptococci into two groups, *Streptococcus faecalis* var. *zymogenes* and *Str. durans*. The only interesting feature about the fermentation reactions is the breakdown of glycerol by all of the strains isolated; with the exception of the strains isolated from Roquefort cheese all of the strains produced acid from glycerol within 24 hr.

DISCUSSION

From this limited survey it appears that the haemolytic streptococci are not peculiar to any type of cheese tested and that all strains are members of the serological group D. They are able to grow in Stilton and Cheddar containing 4–6% salt and Roquefort and Danish Blue containing in the aqueous phase 10–20% salt, a condition unlikely to encourage the growth of the pathogenic streptococci. No significance is attached to the observation made on the few samples tested, that different types of cheese or cheese from different localities may contain different variants of *Str. faecalis* var. *zymogenes* and *Str. durans*.

This would appear to be the first report of the haemolytic *Str. durans* having been isolated from a British dairy product. Abd-el-Malek & Gibson (5) and Shattock (6) reported the isolation from British dried milk powder of strains of group D differing from *Str. durans* only in their inability to haemolyse horse blood in nutrient agar. These strains they believed to be variants of *Str. durans*.

The fermentation of glycerol by *Str. faecalis* var. *zymogenes* and *Str. durans* appears to be unusual. None of the strains of *Str. durans* isolated from milk (Sherman & Niven (7)), dried milk powder (Sherman & Wing (8)) and faeces (Smith & Sherman (9)) fermented glycerol. That all the strains of haemolytic streptococci isolated from cheese ferment glycerol may be an indication of the adaptability of the members of group D to environment, and it would be of interest to know whether the non-haemolytic members of group D in cheese are also able to ferment glycerol. If the fermentation of glycerol is due to adaptation, then either the streptococci are supplied with glycerol by the action of other organisms—for presumably the source of glycerol in cheese is the fat which amounts to about 33% of the weight—or they are themselves lipolytic. In the case of Roquefort, Danish Blue and Stilton cheese the moulds may break down the fat, but the organisms isolated from Cheddar cheese which is not inoculated with mould also ferment glycerol. The lipolytic action of group D streptococci under the anaerobic conditions prevailing in cheese are not known.

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422. CHEESE DISCOLORATION: OXIDATION OF BIXIN IN ANNATTO-COLOURED CHEESES PROMOTED BY SULPHYDRYL COMPOUNDS

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(With 1 Plate)

Some years ago discoloration was reported in mature New Zealand export cheeses. The defect occurred typically as bleached or pink areas surrounding cracks, though a less frequent 'muddy' discoloration was also noted. Discoloration was, on the whole, restricted to cheeses made in warm weather. Preliminary observations showed that the defect had certain characteristic features, viz.

(i) Bleached and pink discolorations were confined to coloured cheeses, whereas the 'muddy' defect was common to both the white and coloured product.

(ii) The defect was observed only in mature cheeses, particularly in those held at relatively high storage temperatures.

(iii) Discoloration was confined to areas adjacent to cracks and slits, being more pronounced in the centre of the defective cheeses. The more open-textured cheeses tended to be more discoloured.

(iv) Bleaching sometimes developed along the cracks of freshly cut cheese surfaces when exposed for several hours to the air.

(v) Bleached areas, on the other hand, sometimes disappeared from cut surfaces when left in the air.

(vi) Defective cheeses, as well as being discoloured and very 'open', invariably had a strong 'sulphide' odour, many being graded as 'stinker' cheeses.

Moir (6) and Morgan (7) noted the occurrence of mould in cracks of discoloured cheeses, and Moir considered that a combination of the actions of mould enzymes, acid and reduction on annatto pigments was responsible for the trouble.

Many discoloured cheeses examined at this Institute, however, had no mould growth along the cracks, and it was shown (1) that discoloration could be produced in cheese by inoculating the milk during manufacture with a small amount of a suspension of discoloured cheese. The results gave strong presumptive evidence that the defect was of bacterial origin. Sherwood (9), while studying the influence of different strains of lactobacilli on cheese flavour, isolated a number of organisms which, inoculated into milk during cheesemaking, promoted discoloration as well as 'unclean' or 'sulphide' odour in the final product.

Meanwhile Barnicoat studied the mechanism of the discoloration changes and concluded that they resulted from oxidation. He found that annatto suspensions on filter-paper, of similar concentrations and pH (4·5–5·0) to those occurring in cheese, were fairly stable towards air, but changed to the typical shades of bleached and pink discoloration

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when dilute hydrogen peroxide was added (3). He postulated the presence in defective cheeses of an oxidation promoter, which preliminary work indicated to be of the nature of a sulphydryl compound.

The association between the 'sulphide' odour of cheeses and their tendency to discolour on exposure to air became even more obvious when experiments were made with tinned products.

The results reported in this paper confirm the view (3) that sulphydryl compounds, well known as pro-oxidants of unsaturated compounds such as linoleic acid (4, 5), are responsible for the destruction of the carotenoid pigment bixin, present in annatto-coloured cheeses, leading to bleached and pink discoloration defects. The curious features of this type of discoloration, as listed in (i)–(vi) of the introductory paragraph, are also explained.

EXPERIMENTAL

Comparative analyses of normal and discoloured portions of cheeses failed to disclose any chemical differences except that (*a*) discoloured portions contained less annatto pigment extractable by 94% methyl alcohol and water mixture (3); (*b*) unaffected (interior) portions of discoloured cheeses, or of cheese which became discoloured after exposure to air, gave more intense responses to the nitroprusside reaction for sulphydryl than did normal cheeses; and (*c*) bleached and pink discoloured areas of defective cheeses gave negative, or only very faint, reactions for sulphydryl compounds. The following observations were also made:

(i) *Changes in annatto pigments due to discoloration.* Bleached and pink discoloration are variants of the same reaction (3). Oxidation of buffered annatto suspensions with low concentrations of hydrogen peroxide gives rise to pink discoloration, while higher concentrations promote bleaching. In experimental cheeses, however, bleaching was invariably the only type of discoloration observed. Although it would be anticipated from the results of experiments with annatto and H_2O_2 that pink discoloration in cheese would be the result of a lower rate of oxidation and hence would differ from bleaching only in degree, it was not found possible to reproduce pink discoloration in cheese under experimental conditions.

The destruction of the annatto pigments, even in the most bleached areas of discoloured cheeses, does not exceed 70% of the original amount. Under these circumstances, fat-soluble carotenoids are also slightly oxidized, and in extremely defective cheeses fat from discoloured portions adjacent to slits also gives evidence of oxidation (fat aldehyde test (8)).

(ii) *Role of oxygen.* The contrast in behaviour between normally matured and tinned cheeses gave clear-cut evidence of the oxidative nature of the chemical change responsible for discoloration and, incidentally, of the unimportance of moulds. Tinned cheeses, made from curd predisposed to discoloration, never appeared discoloured on opening, but developed the defect after a few hours' exposure to the atmosphere. Mature cheeses made from the same batch of curd and cured in the usual way would, on the other hand, exhibit the defect when first cut, though not to the same extent as that finally attained by the tinned product. As previously mentioned, pink discoloration was confined to normally matured cheeses and was much less common than the bleached defect.

(iii) *Sulphydryl compounds in cheese.* The association between discoloration and the 'sulphide' odour in cheeses called for a method of estimating traces of —SH compounds.

Attempts to determine —SH compounds by distillation procedures proved unsuccessful, however, as these substances, present only in minute amounts, are extremely sensitive to oxidation. A rapid colorimetric method of estimation, sensitive to 5 p.p.m. —SH, was finally adopted: 1 g. freshly cut cheese was immediately ground with 0·1 g. powdered 5% sodium nitroprusside in ammonium sulphate, together with 0·5 ml. saturated ammonium sulphate, followed by 0·5 ml. conc. ammonia. The colour was compared with standards made with cysteine hydrochloride. Normal cheeses contain below 35 p.p.m. —SH; undiscoloured portions of defective cheeses 50–100 p.p.m. and even more; undiscoloured areas adjacent to slits (in both discoloured and normal cheeses) contain amounts of —SH similar to the normal interior portions, whereas discoloured areas give only faint, or usually negative, reactions.

Sulphydryl compounds are present both in the protein-residue and protein-free filtrate of cheese extracts made with saturated ammonium sulphate. Treatment of curd proteins with buffered lactic: lactate solutions (hydrolysis) fails to produce sulphydryl compounds, and since a faint positive reaction is first noted in cheeses when graded about 14 days after manufacture, this observation supports the theory that lactobacilli are responsible for the formation of sulphydryl compounds, as these organisms replace lactic streptococci at this stage.

Attempts to identify the sulphydryl compounds proved abortive. Neither methyl mercaptan (which could possibly be derived from methionine), nor thioglycollic acid (the sulphur analogue of lactic acid) added to cheese reproduced the 'stinker' odour, nor did ethyl mercaptan or diethyl sulphide simulate the natural defect. Traces of hydrogen sulphide produced a sharp but relatively inoffensive odour.

Initial experiments were made with glutathione, cysteine and ethyl mercaptan, but hydrogen sulphide, which can conveniently be added at any stage, merely by exposing cheese to H_2S:CO_2 mixtures, was found suitable for demonstrating the principal phenomena of discoloration encountered in practice. For example, buffered annatto suspensions containing traces of sulphydryl derivative exhibit no change in colour until exposed to air, after which a rapid bleaching, accompanied by disappearance of sulphydryl, occurs.

Cheese-curd proteins readily 'fix' sulphydryl from dilute hydrogen sulphide solutions, i.e. disulphide linkages of the amino-acid cystine are reduced to the sulphydryl group of cysteine. The maximum amount of sulphydryl introduced in curd protein by this means appears to be of the order of 900 p.p.m., corresponding to about 250 p.p.m. in a typical Cheddar cheese. The estimated sulphydryl content of even the most defective portions of 'stinker' cheeses rarely exceeded about 100 p.p.m. however.

The relationships between causal organisms, reducing conditions occurring during ripening (including, for example, the production of sulphydryl compounds), oxidation and discoloration in cheeses are shown in Fig. 1.

The four cheeses were made from milk containing approximately 0·4% suspension of a bleached discoloured cheese. The milk was apportioned between two vats: (*a*) coloured with annatto according to the usual practice, and (*b*) not coloured and containing 20 p.p.m. methylene blue as oxidation-reduction indicator.

Loaf cheeses, made by pressing hoops half-filled with curd from each vat, were allowed to mature at different temperature levels for more than 10 months. A photograph (Fig. 1) taken shortly after cutting, and showing a typical selection of the inoculated experimental

cheeses (annatto-coloured half upwards), illustrates a number of the points discussed in this paper, viz.

(*a*) Reducing conditions in mature cheeses responsible for bleaching of methylene blue (and incidentally production of sulphydryl compounds).

(*b*) Oxidation of methylene blue at slits, due to ingress of air, but not at all slits (e.g. lower half of D).

(*c*) Similarity in appearance of bleached areas (upper parts) and oxidized areas of methylene blue around cracks (A, B, D), and actual merging of 'blueing' and bleaching at borderline of curds.

(*d*) Ingress of air into tinned cheese (G) shortly after opening. The lower (methylene blue) portion of G was colourless when first cut, but developed methylene blue oxidation round the slits to the extent shown in the photograph within one hour. This cheese subsequently developed bleached discoloration around the cracks of the normal (upper) portion.

(*e*) Darkening effect of desiccation (compare edges and interiors of A, B, D, and the colour of the moister, tinned cheese (G) with the others).

Table 1. *Pro-oxidative effect of sulphydryl on bixin of coloured cheeses exposed to air*

(Pigment concentrations compared on equal moisture basis.)

	Initial		3 days		7 days	
Original mature cheese	—SH reaction	Relative bixin content	—SH reaction	Relative bixin content	—SH reaction	Relative bixin content
Interior (control)	+ +	100	+ +	100	+ +	100
			Exposed to air			
Small blocks	+ +	100	—	—	Nil	86
Small blocks impregnated with traces H_2S	+ + + +	104	+ +	65	(+)	31

Annatto-coloured cheeses (made from normal or skim milk) containing traces of hydrogen sulphide subsequently added in an inert, humid atmosphere, also appeared normal until exposed to air, when oxidation promoted typical bleached discoloration, though more slowly than was the case with defective tinned cheeses after opening. Table 1 shows results obtained with small blocks of mature, normal cheese giving a normal sulphydryl reaction and typical of a number of experiments. These and similar results reproduced those obtained with normally occurring bleached cheese and show that sulphydryl compounds in cheese are autoxidizable and catalyse the simultaneous oxidation and destruction of the carotenoid pigment bixin derived from annatto; also that apparently normal coloured cheese may actually lose a fraction of its bixin by oxidation when cut, but that the change is usually masked by the darkening effect of desiccation.

DISCUSSION

The following would appear to offer a satisfactory explanation of the mechanism responsible for discoloration of annatto in coloured cheeses associated with 'stinker' (sulphydryl) development:

(i) Lactobacilli capable of producing considerable amounts of volatile sulphydryl

Fig. 1. Cheeses made from milk containing a suspension of discoloured cheese (upper part—with annatto; lower part—uncoloured and containing methylene blue). Cured for 10 months: A, 55° F.; B and D, at 45° F.; G, at 45° F. (tinned).

compounds, and also an open texture (as a result of gas formation), became established in the product during the course of manufacture.

(ii) High temperature conditions during curing and storage favour the growth of these undesirable types of lactobacilli.

(iii) Volatile, soluble sulphydryl compounds formed reduce disulphide (cystine) linkages of curd proteins to sulphydryl groups (cysteine).

(iv) Cracks develop, in many cases leading to openings in the rind, and due to changes in temperature and barometric pressure air is pumped into the cheese.

(v) Sulphydryl compounds being autoxidizable, promote the simultaneous oxidation of the unsaturated groups of the carotenoid bixin (annatto), but because of the darkening caused by desiccation the defect is sometimes masked at the edges and more prominent in the centre of the cheese. (By no means all cracks lead to openings in the rind.)

(vi) Rapid oxidation favours bleached discoloration, and the reasons for a small proportion of the affected cheeses developing pink discoloration, which laboratory experiments suggest as being the result of a milder oxidation are still obscure. The least common 'muddy' discoloration occurred also in 'white' cheeses and is therefore not related to the annatto.

(vii) On exposure to air, bleaching may disappear merely as the result of being masked by the darkening effect of desiccation.

(viii) By carefully extracting annatto pigments with solvent mixtures it is shown that a slight loss of bixin in apparently normal cheeses occurs after exposure to air.

Some loss of bixin in mature coloured cheese on exposure to air appears therefore to be normal, as all such cheeses were found to contain sulphydryl compounds. The defect is apparently one of degree rather than of kind and becomes objectionable only when sulphydryl compounds are present in excessive amounts.

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